(FILE 'HOME' ENTERED AT 15:14:15 ON 12 FEB 2002)

FILE 'USPATFULL' ENTERED AT 15:14:45 ON 12 FEB 2002

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L1           39 S LYSOSTAPHIN AND (SYSTEMIC OR INTRAVENOUS)
L2           34 S L1 NOT (SYSTEMIC (5A) (TOXIC? OR DISEAS?))

=> d bib,kwic 2,4,14,15,19,21,24,29,33
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L2 ANSWER 2 OF 34 USPATFULL

AN 2002:12030 USPATFULL

TI METHOD FOR THE TREATMENT OF STAPHYLOCOCCAL DISEASE

IN GOLDSTEIN, BETH P, TARRYTOWN, NY, UNITED STATES
CLIMO, MICHAEL W, RICHMOND, VA, UNITED STATES
NOVICK, RICHARD P, NEW YORK, NY, UNITED STATES
ARCHER, GORDON L, RICHMOND, VA, UNITED STATES

PI US 2002006406 A1 20020117

AI US 1998-120030 A1 19980721 (9)

PRAI US 1997-53470 19970723 (60)

DT Utility

FS APPLICATION this case

LREP WHITE & CASE LLP, PATENT DEPARTMENT, 1155 AVENUE OF THE AMERICAS, NEW YORK, NY, 10036

CLMN Number of Claims: 31

ECL Exemplary Claim: 1

DRWN 2 Drawing Page(s)

LN.CNT 808

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB **Lysostaphin** is demonstrated to be a powerful anti-staphylococcal agent suitable for parenteral administration to mammals including humans. Low dosages, on the order of 0.5 - 45 mg/kg/day are sufficient to eradicate most staphylococcal infections. **Lysostaphin** is also effective against bacteria of this type which have developed resistance to conventional antibiotics such as penicillins and vancomycin. **Lysostaphin** analogues, such as variants and related enzymes, show similar activity.

SUMM [0003] This invention pertains to the administration of **lysostaphin** or the purpose of treatment of staphylococcus infection in mammals, including humans, as well as pharmaceutical preparations used in said. . . . bacteremia; and staphylococcal infection of kidneys, lungs, skin, bone, burns, wounds and prosthetic devices. The invention embraces the use of **lysostaphin** broadly, including not only wild type **lysostaphin** but recombinant **lysostaphin**; **lysostaphin** variants with amino acid sequences varying from the published `natural sequence` of the mature peptide (U.S. Pat. No. 4,931,390) due. . .

SUMM [0005] **Lysostaphin** is an enzyme, first identified in Staphylococcus simulans (formerly known as S. staphylolyticus), which has antimicrobial activity by virtue of. . . on glycine-containing bridges in the cell wall peptidoglycan of bacteria [Zygmunt, et al., Progr. Drug Res. 16:309-333 (1972)]. In vitro, **lysostaphin** is particularly active against Staphylococcus aureus, because the cell wall bridges of this species contain a high proportion of glycine,. . .

SUMM [0006] The activity of **lysostaphin** has also been examined in animal infection models. Studies in which intraperitoneal treatment followed intraperitoneal infection are similar to in. . . subjected to intraperitoneal infection followed by single or multiple subcutaneous administrations with a total of approximately 1 mg/kg of a **lysostaphin** preparation [Schuhardt, et al., J. Bacteriol. 88:815-816 (1964); Harrison, et al., Can. J. Microbiol. 13:93-97 (1967)]. A total dosage of. . .

SUMM [0008] When a **lysostaphin** preparation was administered

intravenously within 6 hours after infection, significant reductions in the numbers of bacteria in the kidneys were. . . seen when treatment was withheld for 24 hours or longer, even with dosages of 125 or 250 mg/kg of a **lysostaphin** preparation. The effect of multiple treatments was not studied.

SUMM . . . The Goldberg, et al. experiment was not comparative, and is therefore of limited utility in assessment of the administration of **lysostaphin**. However, high dosages of **lysostaphin** (at least 50 mg/kg/treatment) were only moderately effective, as judged by the health of the dogs and by the extent. . .

SUMM [0010] Accordingly, the data obtained from prior art studies with animal models do not teach that use of **lysostaphin** would be an effective and practical approach to clearing established infections from various organs.

SUMM [0011] Limited human trials were conducted aimed at eradication of nasal carriage of S. aureus by topical application of **lysostaphin** to the nares [Martin, et al., J. Lab. Clin. Med. 70:1-8 (1967); Martin, et al., J. Lab. Clin. Med. 71:791-797. . .

SUMM [0012] The art reports treatment of one very ill human patient with a single dose of parenterally administered **lysostaphin**, followed by an antibiotic, gentamicin, three days later. The patient died, but did exhibit a reduction in bacteremia [Stark, et. . .

SUMM . . . phenomena observed during the course of the animal and human studies, were noted as a great concern. Contamination of the **lysostaphin** preparations with extraneous substances may have been responsible for at least some of these phenomena.

SUMM . . . of desired effectiveness in the studies discussed. This may have been further due to the difficulty in producing and purifying **lysostaphin**.

SUMM [0015] The staphylococcal gene for **lysostaphin** has now been sequenced and cloned [U.S. Pat. No. 4,931,390]. Lyostaphin for use as a laboratory reagent has been produced. . .

SUMM [0017] The administration of relatively low dosages of **lysostaphin** (under 50 mg/kg) via parenteral administration is a dramatically effective therapy for the treatment of staphylococcal infections, particularly infections that are resistant to treatment, and/or typically associated with significant morbidity and mortality. Further, **lysostaphin** is demonstrated to be effective against staphylococcal bacteria that are at least partially resistant to available antimicrobial agents, such as. . .

SUMM [0018] The invention further includes combination therapies comprising alternating or simultaneous administration of **lysostaphin** and one or more other antimicrobial agents. Particularly preferred antibiotics for administration in concert with **lysostaphin** according to this invention are rifamycins (isolated from microorganisms or synthetically or semi-synthetically produced, such as rifampin) and glycopeptides (a. . .

SUMM [0019] The availability of cloned, recombinant and variant **lysostaphins** further expands this invention. Related enzymes have been identified, and can further be used together with, or in place of, **lysostaphin**.

SUMM [0020] The cloning and sequencing of the **lysostaphin** gene permits the isolation of variant enzymes that can have properties similar to or different from those of wild type **lysostaphin**. One such altered enzyme, bearing a single amino acid change and which was the result of our work, has been. . .

SUMM [0021] Other **lysostaphin** analogues, including naturally occurring enzymes with sequence homology to lypostaphin and with endopeptidase activity, or even chimeric enzymes obtained by. . .

DRWD [0022] FIG. 1 is a graphical representation of the bactericidal activity of **lysostaphin** against a methicillin-resistant S. aureus strain, as compared with vancomycin.

DRWD [0023] FIG. 2 is a graph reflecting the bactericidal activity of **lysostaphin** against a variety of S. aureus strains of differing antimicrobial resistance.

DRWD [0025] **Lysostaphin** analogue--Any enzyme, including **lysostaphin** (wild type), any **lysostaphin** mutant or variant, any recombinant, or related enzyme that retains the proteolytic ability, in vitro and in vivo, of proteolytic. . . the process) or by mutation of the structural gene. Mutations may include site-deletion, insertion, domain removal and replacement mutations. The **lysostaphin** analogues contemplated in the instant invention may be recombinantly expressed or otherwise.

DRWD [0026] Parenteral--Administration by injection, including **intravenous**, intramuscular, subcutaneous, intraorbital, intraspinal, intraperitoneal and by direct perfusion or delivery to organs or tissues through injection (e.g., intramedullary).

DETD [0030] **Lysostaphin** has been found to be highly active, at moderate doses. This is demonstrated, below, in a very severe well-characterized animal. . . not seen with currently available antimicrobial agents. We further demonstrate herein that combination of an even lower daily dosage of **lysostaphin** with a standard therapeutic agent potentiates the antimicrobial activity of the components in this model system.

DETD [0031] The **lysostaphin** dosages we used were significantly lower than those previously demonstrated to have only a limited effect on clearance of bacteria. . .

DETD . . . also demonstrated, below, activity against staphylococci, in vitro and in a mouse acute infection model, of an altered form of **lysostaphin**, generated by mutagenizing a recombinant strain of Bacillus sphaericus carrying the **lysostaphin** gene. It is therefore another realized aspect of the invention to administer pharmaceutical preparations of **lysostaphin** analogues, either **lysostaphin** or other enzymes with peptidoglycan endopeptidase activity, including genetically modified enzymes containing one or up to five amino acid substitutions;. . .

DETD [0033] For example, another glycylglycine endopeptidase (ALE-1, from Staphylococcus capitis EPK1) has been described. ALE-1 is distinct from **lysostaphin**, although the two enzymes have considerable amino acid homology [Sugai et al., J. Bacteriol. 179:1193-1202(1997)]. Another peptidoglycan hydrolase with a lower degree of homology to **lysostaphin**, but which also possesses endopeptidase activity, is zoocin A, produced by Streptococcus zooepidemicus 4881 [Simmonds et al., Applied and Environmental. . . proteins can be produced by the fusion of a domain of these or similar enzymes to a domain of a **lysostaphin** analogue.

DETD . . . may give concern in some, but not other situations (such as emergency or short term situations) suitably pure preparations of **lysostaphin** analogues, obtained by the fermentation of harmless recombinant strains of bacteria, are expected to be less prone to induce immunogenic. . .

DETD . . . solutes for osmotic balance) for reconstitution with liquids, suitable for parenteral delivery of the active agent. Delivery is preferably via **intravenous** (i.v.), intramuscular (i.m.), subcutaneous (s.c.), or intraperitoneal (i.p.) routes or intrathecally or by inhalation or by direct instillation into an. . .

DETD [0036] Furthermore, the active **lysostaphin** analogue can be coadministered, simultaneously or alternating, with other antimicrobial agents so as to more effectively treat an infectious disease.. . . be in, or reconstituted in, a larger volume to be administered by slow i.v. infusion. Agents to be coadministered with **lysostaphin** or other antibacterial enzymes may be formulated together with said enzyme as a fixed combination or may be used extemporaneously. . .

DETD [0037] Suitable dosages and regimens of **lysostaphin** may vary

with the severity of the infection and the sensitivity of the infecting organism and, in the case of. . .

DETD [0038] All experiments were conducted using **lysostaphin** analogues produced by fermentation of recombinant B. sphaericus strains engineered to contain the **lysostaphin** gene described by Recsei (U.S. Pat. No. 4,931,390) or a mutant thereof. Specifically, the **lysostaphin** analogues prepared by fermentation of B. sphaericus varied from the published sequence by having as many as 2 fewer or. . .

DETD [0039] In particular, the data herein are largely derived from studies using preparations of recombinantly produced **lysostaphin** analogues wherein the majority component is one that lacks the two N-terminal amino acids of the published sequence. However, the. . .

DETD [0040] In Vitro Activity of **Lysostaphin**

DETD [0041] As shown in Table 1a, experiments demonstrated that the **lysostaphin** preparation was active and bactericidal in vitro against clinical isolates of S. aureus; the minimal inhibitory concentrations (MIC) and minimal. . .

DETD [0042] Furthermore, **lysostaphin** was shown to be active against a number of isolates of Staphylococcus epidermidis (a coagulase-negative species) with MIC .ltoreq.8 .mu.g/ml. . . concentration that killed 99.9% of the initial inoculum in 24 hours of exposure. As shown in Table 1a, susceptibility to **lysostaphin** is not affected by resistance or reduced sensitivity to methicillin and/or vancomycin. S. aureus strains that are methicillin-resistant, and also. . . Control and Prevention, Morbidity and Mortality Weekly Report 1997. 46:813-815].

TABLE 1a

Preliminary study of in vitro susceptibility of S. aureus to **lysostaphin**

Strain	MIC (.mu.g/ml)	MBC (.mu.g/ml)
1573.sup.c,m	0.5	1
27619.sup.c,m	0.25	0.5
COL.sup.c,m	0.13	0.25
450M.sup.c,m	0.25	0.5
402.sup.c	0.5	0.5

. . .

DETD [0043] **Lysostaphin** sticks to plastic materials and can be lost from solution; this can affect its apparent activity. Therefore, some MIC determinations. . . Otherwise, the method was identical to that cited above. As shown in table 1b , the in vitro activity of **lysostaphin** against the strains tested improved by 8- to 64-fold when tested in the presence of BSA. Since this observation is related to the affinity of **lysostaphin** for plastic materials, it is to be expected that, in general, staphylococcal strains are more susceptible to **lysostaphin** than was observed previously.

TABLE 1b

Activity of **lysostaphin** against S. aureus with and without BSA

Strain	MIC (.mu.g/ml) With BSA	MIC (.mu.g/ml) Without BSA
417	0.03	
414	0.03	0.25

. . .

DETD [0044] These data demonstrate the very potent activity of **lysostaphin** against contemporary clinical isolates of multiply antibiotic-resistant Staphylococcus aureus.

DETD [0045] The bactericidal activity of **lysostaphin** against S. aureus was also studied by means of time-kill experiments. In one experiment of this type, S. aureus strain. . . w/v.) The plates were incubated for 24-48 hours at 36.degree. C. and the colonies were counted manually. All dilutions of **lysostaphin** were made in the presence of 0.1-0.2% BSA, to prevent adsorption of **lysostaphin** to plastic materials. Vancomycin (Sigma Chemical Co.) was diluted in sterile distilled water.

DETD [0046] As shown in FIG. 1, **lysostaphin** at concentrations of 0.004 and 0.032 .mu.g/ml was rapidly bactericidal, with at least 99.9% of the bacteria being killed within. . . hours of contact, even though much higher concentrations of vancomycin (2 and 16 .mu.g/ml) were used. The different concentrations of **lysostaphin** and vancomycin used were one and eight times their respective MIC.

DETD . . . stage of growth. As indicated in FIG. 2, the bacterial titers ranged from 2.times.10.sup.6 to 9.times.10.sup.7 CFU/ml at this time. **Lysostaphin** was added to each culture at the concentration of 1 .mu.g/ml. At intervals, samples were withdrawn, serially diluted in 0.9%. . . C. and the colonies were counted manually. As shown in FIG. 2, all of these strains were rapidly killed by **lysostaphin**.

DETD [0048] These data demonstrate that **lysostaphin** has potent and rapid bactericidal activity against contemporary clinical isolates of S. aureus, including strains resistant to methicillin and strains. . .

DETD [0049] Comparative efficacy of **lysostaphin** in a mouse S. aureus infection model

DETD [0050] The efficacy of **lysostaphin** was compared to that of vancomycin in an acute infection model in mice. S. aureus Smith was cultured overnight, with. . . tenfold the inoculum that reproducibly killed all untreated animals within 48 h. There were six mice in each treatment group. **Lysostaphin** was administered intravenously (in 0.1 ml 5% dextrose for injection) or subcutaneously (in 0.2 ml), within 10 min of infection.. . .

DETD [0051] As shown in Table 2, **lysostaphin** protected 100% of the infected mice when given at a dosage of 0.16 mg/kg intravenously or at a dosage of. . . at the dosage of 2.5 mg/kg. All of the untreated mice died in less than 24 hours.

TABLE 2

Efficacy of **lysostaphin** against S. aureus infection in mice

Dose (mg/kg)	% survival **lysostaphin** iv	**lysostaphin** sc	vancomycin
0	0	0	0
0.08	33		
0.16	100		
0.31	100		
0.63	100		0
1.25		67	83
2.5		100	100
5		100	100
10		100	
20		100	

DETD [0052] This example demonstrates that **lysostaphin** is effective against S. aureus infection in an acute infection model in mice using a highly virulent challenge dose of bacteria. When administered intravenously, exceedingly low doses of purified recombinant **lysostaphin** were effective. On a weight basis, **lysostaphin** was 16 times as effective as vancomycin; on a molar basis, **lysostaphin** was about 200 times as effective as

vancomycin.

DETD [0053] In vitro and in vivo activity of a variant **lysostaphin** enzyme

DETD [0054] A Bacillus sphaericus strain containing the cloned **lysostaphin** gene described in U.S. Pat. No. 4,931,390 was mutagenized with N,N-nitrosoguanidine. Surviving colonies were screened for presence of a lytic. . .

DETD [0055] One of these clones was further characterized. The **lysostaphin** gene was sequenced and found to contain a single G-to-A mutation in the codon corresponding to position 218 of the mature **lysostaphin** protein, resulting in a codon change from GGT (glycine) to GAT (aspartic acid). Fermentation of this mutant strain produced sufficient. . .

DETD . . . As shown in table 3, the variant enzyme was highly active against S. aureus in vitro, although the wild type **lysostaphin** preparation was somewhat more active. In this experiment, MICs were determined by broth macrodilution in 1 ml final volumes in glass tubes. Otherwise, the methodology was as described above.

TABLE 3

Activity of variant **lysostaphin** against S. aureus in vitro

	MIC (.mu.g/ml)			
	AG417	AG404.sup.c,m	AG402.sup.c	AG414
Gly218Asp	.03	.06	.06	.03
wild type **lysostaphin**	.004	.008	.008	.004

.sup.cclinical isolate; .sup.mmethicillin-resistant

DETD [0057] As shown in table 4, the variant **lysostaphin** enzyme was also highly active against S. aureus in the acute mouse infection model. Here again, the variant was somewhat less active than the wild type **lysostaphin**, but it was more active than vancomycin.

TABLE 4

Activity of variant **lysostaphin** against S. aureus infection in mice.

	% survival			
Dose (mg/kg)	Control	**Lysostaphin**	Gly218Asp	Vancomycin
0	0			
0.04		0		
0.08		17	0	
0.16		83	17	
0.31			33	
0.63			83	0
1.25				83
2.5				100

DETD [0058] Antimicrobial activity in the serum of a rabbit treated with **lysostaphin**.

DETD [0059] A New Zealand white rabbit weighing approximately 5 kg was given an **intravenous** infusion of 125 mg **lysostaphin**. Blood samples were taken at intervals up to 4 h and serum was prepared; two-fold serial dilutions were made, and. . .

DETD [0060] As shown in table 5, the serum contained highly bactericidal concentrations of **lysostaphin** over the entire period of time. In particular, at time points from 30 minutes to 120 minutes, the titer was. . . 99.9% of the bacteria. At the latest time point, 240

minutes, the titer was 1:64.

TABLE 5

Serum bactericidal titer of **lysostaphin** after administration of 125 mg to a 5-kg rabbit

Time after beginning of infusion (minutes)	Serum bactericidal titer
0	1:128

. . .

DETD [0061] This example demonstrates that **lysostaphin** maintains bactericidal activity in the serum of rabbits and that it remains present and active in the circulation for at. . .

DETD [0062] Efficacy of **lysostaphin** against experimental endocarditis in rabbits.

DETD . . . animals were randomly assigned to different treatment groups; untreated control (9 rabbits); positive control, vancomycin 30 mg/kg twice daily (15); **lysostaphin** 5 mg/kg three times daily (11); **lysostaphin** 5 mg/kg once daily (10); **lysostaphin** 5 mg/kg once daily+vancomycin 30 mg/kg twice daily (11). Any rabbits whose infection was not confirmed by pre-treatment blood culture. . .

DETD [0064] All treatments were **intravenous** and were carried out for three days. The state of health of the rabbits was assessed at intervals. The rabbits. . .

DETD [0066] As shown in table 6, a regimen of 5 mg/kg **lysostaphin** three times daily was the most efficacious treatment. An impressive statistic is that this treatment completely sterilized the heart valve. . . used as a positive control in this infection model: 30 mg/kg of vancomycin twice daily. A regimen of 5 mg/kg **lysostaphin** once daily was less efficacious than the thrice daily regimen, but was almost as good as vancomycin in reducing bacterial counts in the vegetation; in fact, the effect was not statistically different from the vancomycin group. The once-daily **lysostaphin** regimen also achieved complete sterilization of the vegetations in some animals. The addition of **lysostaphin** once daily to the standard vancomycin regimen produced a dramatic lowering in mean bacterial count, almost to the level seen with 3 daily **lysostaphin** treatments. However, in terms of the number of vegetations completely sterilized, the three-times-daily **lysostaphin** regimen was clearly superior to all others.

TABLE 6

Efficacy of **lysostaphin** against S. aureus endocarditis in rabbits

Treatment	Mean log.sub.10CFU/gram of vegetation .+-. standard deviation		Number of sterile vegetations/ total animals treated
Untreated control	10.73	.+-. 1.58	0/9
Vancomycin 30 mg/kg twice daily	5.91	.+-. 1.67.sup.a	0/15
Lysostaphin 5 mg/kg once daily	7.08	.+-. 3.74.sup.a	2/10
Lysostaphin 5 mg/kg three times daily	2.26	.+-. 0.85.sup.a,b	10/11.sup.c

Lysostaphin 5 mg/kg once daily + vancomycin 30 mg/kg twice daily 3.23 .+-. 1.41.sup.a,b 3/11

.sup.ap < 0.05 compared to untreated control; .sup.bp < 0.05 compared to vancomycin;
.sup.cp = 0.008 vs **lysostaphin** once daily + vancomycin

DETD [0067] Kidney abscesses were also assessed for the presence of staphylococci. The thrice-daily regimen of **lysostaphin** dramatically reduced the bacterial load as compared with the untreated control group to just over 10.sup.2 CFU/gram of tissue in the **lysostaphin** group as compared with just under 10.sup.8 CFU/gram in the controls.

DETD [0068] Observation of the animals demonstrated that rabbits treated with the thrice-daily regimen of **lysostaphin** were all in good health early in the treatment cycle.

DETD . . . agent in this infection model. The fact that sterilization occurred within a relatively short treatment period, 3 days, indicates that **lysostaphin** acts very rapidly in vivo and suggests that antimicrobial **lysostaphin** analogues could greatly improve the outcome in patients with serious staphylococcal infections that require rapid reduction in bacterial load.

DETD [0070] The above data demonstrate the efficacy of **lysostaphin** analogues against S. aureus, including MRSA (methicillin-resistant S. aureus). Strains that are both methicillin-resistant and resistant to vancomycin are a. . .

DETD [0071] As shown in table 7, **lysostaphin** was efficacious in treating rabbits with infective endocarditis caused by the methicillin-resistant VISA strain.

TABLE 7

Efficacy of **lysostaphin** against endocarditis in rabbits caused by a methicillin-resistant VISA strain of S. aureus

Treatment	CFU/g vegetation*	sterile/total vegetations
Control	10.3	0/10
Vancomycin 30 mg/kg twice daily	6.95	0/13
Lysostaphin 5 mg/kg three times daily	6.29	2/10
Lysostaphin 15 mg/kg twice daily	4.0**	0/5

*expressed as log10 of the mean.
significantly better than vancomycin or the lower dose of **lysostaphin (p < 0.05)

DETD [0072] Against the VISA strain, **lysostaphin** at 5 mg/kg three times daily was as effective as vancomycin in reducing the bacterial load in aortic vegetations. **Lysostaphin** at 15 mg/kg twice daily was more effective than the standard dosage regimen of vancomycin (statistically significant) and also was significantly more effective than **lysostaphin** at 5 mg/kg given three times daily. Furthermore, vancomycin, even at 30 mg/kg twice daily, could not achieve complete sterilization. . . . test animals. On the other hand complete sterilization was achieved in some animals with the three times daily regimen of **lysostaphin**.

DETD . . . is accepted as a rigorous test of the ability of antimicrobial agents to cure severe human infections. Previous work with **lysostaphin** in established infections showed limited reduction

in kidney bacterial load in a mouse model and in heart valves and other. . . the rapid, total sterilization of virtually all heart valve vegetations, as has now been seen using very moderate doses of **lysostaphin** in the rabbit endocarditis model.

DETD [0074] The results presented herein demonstrate not only the unexpected effectiveness of **lysostaphin** against S. aureus endocarditis, but show that such efficacy is far superior to that expected for standard treatments. Currently available. . . to prevent such damage as well as metastatic spread of infection to other vital organs. The above results indicate that **lysostaphin** analogues, alone or in combination with other agents, have the potential for effectiveness in the treatment of such infections.

DETD [0075] Furthermore, based on these results and on the in vitro activity of **lysostaphin** against staphylococci, it is to be expected that **lysostaphin** analogues, alone or in combination with other agents, will be useful against species of staphylococci other than S. aureus. Among the agents suitable for use together with **lysostaphin** are vancomycin and other glycopeptides, rifampin and other rifamycins, and other anti-infective agents that have activity against staphylococci.

DETD [0076] **Lysostaphin** analogues may be used not only in the treatment of staphylococcal endocarditis but other potentially lethal staphylococcal diseases, such as. . . type or severity requiring prolonged treatment with currently used antimicrobial agents. The instant invention further extends to the use of **lysostaphin** analogues in treating such infections and diseases when they are caused by staphylococci that are resistant to routinely used antibiotics.

CLM What is claimed is:

. . . . method of treating staphylococcal infection in a mammal, comprising administering to the mammal an effective amount of at least one **lysostaphin** analogue.

2. The method of claim 1, wherein the **lysostaphin** analogue(s) is administered together with at least one other antimicrobial agent.

. . . . mammal suffering from at least one of said disease conditions; and administering to the mammal an effective amount of a **lysostaphin** analogue.

. . . . device, comprising selecting a mammal suffering from such an infection; and administering to the mammal an effective amount of a **lysostaphin** analogue.

6. The method of claim 1, 4 or 5 wherein the **lysostaphin** analogue is **lysostaphin** or a variant thereof which exhibits the biological activity of proteolytic attack against glycine-containing bridges in the cell wall peptidoglycan. . .

10. The method of claim 7, wherein the analogue is **lysostaphin** .

11. The method of claim 8, wherein the analogue is **lysostaphin** .

12. The method of claim 9, wherein the analogue is **lysostaphin** .

14. The method of claim 1, 4 or 5, wherein the staphylococcal infection is at least partially resistant to an antimicrobial agent other than **lysostaphin**.

17. The method of claim 1, 4 or 5 wherein the **lysostaphin** analogue is recombinantly produced.

18. The method of claim 17 wherein the analogue is **lysostaphin**
.

21. The method of claim 4 or 5, wherein the **lysostaphin** analogue is administered together with at least one other antimicrobial agent.

28. A therapeutic composition for the treatment of staphylococcal infection, comprising a **lysostaphin** analogue having the biological activity of proteolytic attack against glycine-containing bridges in the cell wall peptidoglycan of staphylococci and a. . .
31. The composition of claim 28, wherein the **lysostaphin** analogue is recombinantly produced.

L2 ANSWER 4 OF 34 USPATFULL
AN 2001:202193 USPATFULL
TI Topical **lysostaphin** therapy for staphylococcus ocular infections
IN O'Callaghan, Richard J., Slidell, LA, United States
PA Board of Supervisors of Louisiana State University and Agricultural and Mechanical College, Baton Rouge, LA, United States (U.S. corporation)
PI US 6315996 B1 20011113
AI US 1999-289684 19990409 (9)
DT Utility
FS GRANTED
EXNAM Primary Examiner: Achutamurthy, Ponnathapu; Assistant Examiner: Tung, Peter P.
LREP Davis, Bonnie J., Runnels, John H.
CLMN Number of Claims: 14
ECL Exemplary Claim: 1
DRWN No Drawings
LN.CNT 546
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
TI Topical **lysostaphin** therapy for staphylococcus ocular infections
AB A method has been discovered for using **lysostaphin** as an effective antibiotic for topical treatment of Staphylococcus corneal infections (keratitis). **Lysostaphin** applied topically to the cornea by eye drops killed bacteria within the cornea; **lysostaphin** reduced the number of bacteria from approximately 10,000,000 viable bacteria colony forming units ("CFU") in the untreated eye to essentially no viable bacteria in the treated eyes. Treatment by **lysostaphin** was more potent than any of the smaller antibiotics that have been previously tested (e.g., tetracyclines, erythromycins, cephalosporins, vancomycin, aminoglycosides, or fluoroquinolones). Moreover, topical application of **lysostaphin** was effective against the highly antibiotic-resistant Staphylococcus strains.
SUMM . . . stroma, and endothelium) that maintain mechanical integrity, proper hydration, and transparency for adequate vision. Because the cornea is not vascularized, **systemic** drugs do not readily permeate the cornea and are generally not used for therapy of ocular bacterial infections. Topical application. . .
SUMM **Lysostaphin**, a protein of 27,000 Daltons, is a bacterial endopeptidase that is highly lethal to S. aureus and S. epidermidis. It was initially isolated from a strain of Staphylococcus simulans. See C. A. Schindler et al., "**Lysostaphin**: A new bacteriolytic agent for the staphylococcus," Proc.N.A.S., vol. 51, pp. 414-421 (1964); C. A. Schindler et al., "Purification and properties of **lysostaphin** -a lytic agent for Staphylococcus aureus," Biochim. Biophys. Acta, vol. 97, pp. 242-250 (1996); W. A. Zygmunt et al., "In vitro effect of

lysostaphin, neomycin, and bacitracin on Staphylococcus aureus," Canadian Journal of Microbiology, vol. 12, pp. 204-206 (1966); W.A. Zygmunt et al., "Lytic action of **lysostaphin** on susceptible and resistant strains of Staphylococcus aureus," Canadian Journal of Microbiology, vol. 13, pp. 845-853 (1967); W. A. Zygmunt et al., "Susceptibility of coagulase-negative Staphylococcus to **lysostaphin** and other antibiotics," Applied Microbiology, vol. 16, pp. 1168-1173 (1968); W. A. Zygmunt et al., "**Lysostaphin**: Model for a specific enzymatic approach to infectious disease," Progress in Drug Research, vol. 16, pp. 309-333 (1972); and H. P. Browder et al., "**Lysostaphin**: Enzymatic mode of action," Biochemical and Biophysical Research Communications, vol. 19, pp. 383-389 (1965).

SUMM **Lysostaphin** has been shown to be effective in lowering S. aureus infections located internally (e.g., mastitis in mammary glands and aortic valve endocarditis) when **lysostaphin** was injected systemically or into the infected tissues. See A. J. Bramley et al., "Effects of **lysostaphin** on Staphylococcus aureus infections of the mouse mammary gland," Research in Veterinary Science, vol. 49, pp. 120-121 (1990); E. R. Oldham et al., "**Lysostaphin**: Use of a recombinant bactericidal enzyme as a mastitis therapeutic," J. Dairy Sci., vol. 74, pp. 4175-4182 (1991); and M. W. Climo et al., " **Lysostaphin** treatment of experimental methicillin-resistant Staphylococcus aureus aortic valve endocarditis," Antimicrobial Agents and Chemotherapy, vol. 42, pp. 1355-1360 (1998). Topical application of **lysostaphin** has been used to treat S. aureus attached to nasal epithelial cells in the nares. See R. R. Martin et al., "The selective activity of **lysostaphin** in vivo," Journal of Laboratory and Clinical Medicine, vol. 70, pp. 1-8 (1967); K. E. Quickel, Jr., et al., "Efficacy and safety of topical **lysostaphin** treatment of persistent nasal carriage of Staphylococcus aureus," Applied Microbiology, vol. 22, pp. 446-450 (1971); and R. Aly et al.,. . . to nasal epithelial cells," Journal of Infectious Diseases, vol. 141, pp. 463-465 (1980). However, it has not been suggested that **lysostaphin** be applied topically to cross membranes to reach bacteria located inside the body.

SUMM **Lysostaphin** has also been found to be effective against MRSA strains by in vitro culture. It was more effective than the. . .

SUMM The **lysostaphin** gene has been sequenced and cloned. See P. A. Recsei et al., "Cloning, sequence, and expression of the **lysostaphin** gene from Staphylococcus simulans," Proc. Natl. Acad. Sci. USA, vol. 84, pp. 1127-1131 (1987).

SUMM U.S. Pat. No. 3,398,056 describes a process of producing **lysostaphin** by fermentation.

SUMM U.S. Pat. No. 3,594,284 describes a process of producing **lysostaphin** by a reduced fermentation period using a cyclic process.

SUMM U.S. Pat. No. 4,980,163 describes a broad range bacteriocin composition, comprising **lysostaphin** and a lanthionine-containing bacteriocin.

SUMM I have discovered that **lysostaphin**, despite its large size, is an effective antibiotic for topical treatment of Staphylococcus corneal infections (keratitis). **Lysostaphin** can be used in an eye drop medication effective for treating common forms of Staphylococcus eye infections, including some of the most antibiotic-resistant forms. **Lysostaphin** therapy resulted in rapid bacterial killing without any irritation or toxicity associated with its ocular use. The penetration of **lysostaphin** into live corneal tissue was surprising because **lysostaphin** is much larger than antibiotics commonly used to treat corneal infections. **Lysostaphin** applied topically to the cornea by drops killed bacteria within the cornea; **lysostaphin** reduced the number of bacteria from approximately 10,000,000 viable bacteria colony forming units ("CFU") in the untreated

eye to essentially no viable bacteria in the treated eyes. Treatment by **lysostaphin** was more potent than any of the smaller antibiotics that have been previously tested (e.g., tetracyclines, erythromycins, cephalosporins, vancomycin, aminoglycosides, or fluoroquinolones). Moreover, topical application of **lysostaphin** was effective against the highly antibiotic-resistant MRSA strains.

SUMM Two advantages of **lysostaphin** over current antibiotics used for topical therapy are the high potency of **lysostaphin** and its effectiveness on strains resistant to other antibiotics. Another advantage is that the drug lacks the toxicity inherent in. . .

DETD **Lysostaphin** Treatment of Staphylococcus keratitis

DETD To determine the efficacy of **lysostaphin** treatment of methicillin-resistant and methicillin-sensitive Staphylococcus aureus keratitis, a rabbit model was used. The rabbit model is a standard technique. . . divided into three groups: 12 corneas to be treated with 5% aqueous vancomycin; 12, to be treated with 0.28% aqueous **lysostaphin;** and 12, as an untreated control group. Each of the three groups were then divided into an early treatment group. . .

DETD TABLE 1

Antibiotic Treatment of Experimental Keratitis
(MRSA strain 301)

Treatment	Early Therapy (Log CFU)	Late Therapy (Log CFU)
Lysostaphin (0.28%)	0.00 .+-. 0.00	0.85 .+-. 0.46
Vancomycin (5%)	2.30 .+-. 0.85	5.83 .+-. 0.16
Untreated	6.52 .+-. 0.10	6.59. . .

DETD With early therapy (beginning at 4 hr post-infection), **lysostaphin** sterilized all MRSA 301-infected corneas, while untreated corneas contained 6.52 log CFU/cornea (P.ltoreq.0.0001). No MRSA 301-infected corneas treated with vancomycin became sterile; these corneas retained 2.3.+-.0.85 log CFU/cornea. When therapy was begun later (10-15 hours post-infection), **lysostaphin** reduced the CFU/cornea of MRSA 301 to 0.85.+-.0.46 log CFU/cornea, compared to 6.59.+-.0.12 log CFU/cornea of the untreated group (P.ltoreq.0.0001).. . .

DETD Moreover, with early therapy, **lysostaphin** reduced the CFU/cornea of the ISP546 strain to 0.58.+-.0.34 log CFU/cornea compared to 5.94.+-.0.24 log CFU/cornea of the untreated group. . .

DETD Staphylococcus keratitis has been successfully treated with topical drops of **lysostaphin. Lysostaphin** killed MRSA strains replicating in the cornea significantly better than did vancomycin, and also killed non-replicating Staphylococcus in the cornea. . .

DETD . . . including isolates of MRSA strains known to be resistant to fluoroquinolones. Nearly all strains of S. aureus were susceptible to **lysostaphin** at concentrations less than 1 .mu.g/ml; the MIC for MRSA strains was 0.04 .mu.g/ml, a value nearly 50-fold lower than. . .

DETD **Lysostaphin** is more effective than any currently available drug for treating Staphylococcus keratitis. When applied as a single topical drop (0.3%) every 30 min from 4 to 9 hr post-infection, or from 10 to 15 hr post-infection, **lysostaphin** caused significant reductions in the number of Staphylococcus CFU per cornea.

DETD Penetration of **lysostaphin** into the cornea

DETD **Lysostaphin** was shown to penetrate the cornea in the following experiments. Rabbit eyes were infected with S. aureus and then treated. . .

DETD In a second experiment, the effect of **lysostaphin** during post-treatment processing of the corneas was inhibited. Rabbit eyes infected with S. aureus were treated with **lysostaphin** as described in Example 1, except that during the processing of the corneas all **lysostaphin** activity was inhibited by adding zinc ions. Corneas were washed in situ with 50 mM Tris HCl buffer, pH 7.5,. . .

mM ZnCl. Immediately on harvesting, the corneas were placed into the same buffer. Zinc ion is a known inhibitor of **lysostaphin,** preventing its anti-bacterial action. Thus little bacterial killing due to **lysostaphin** could occur during the harvesting and processing of the corneas. The **lysostaphin**-treated corneas were again free of viable bacteria while untreated corneas, also washed in situ and placed in a zinc solution,. . . .

DETD Without wishing to be bound by this theory, it is believed that despite its size **lysostaphin** penetrated the cornea aided by its proteolytic activity as a zinc metalloproteinase, as described by Park et al., "Binding and Degradation of Elastin by the Staphylolytic Enzyme **Lysostaphin,**" Int. J. Biochem. Cell Biol., vol. 27, pp. 139-146 (1995).

DETD No inflammatory or toxic reaction to **lysostaphin**

DETD . . . important question for any new drug treatment is whether the drug will elicit an inflammatory response. The topical application of **lysostaphin** caused no inflammatory or toxic reactions in the rabbit eye. For rabbit eyes infected and treated as in Example 1, there were no differences in the SLE scores of the **lysostaphin** -treated eyes versus untreated and uninfected eyes ("normal" eyes). The SLE scores of **lysostaphin**-treated eyes were identical to those treated with an equal number of applications of water or buffered saline. In contrast, eyes. . . .

DETD Interaction of **lysostaphin** with other antibiotics

DETD **Lysostaphin** is an extremely potent killer of S. aureus, but lacks activity on Gram-negative bacteria and other microbial agents (e.g., acid-fast bacteria). **Lysostaphin** therapy has a potential to be used in conjunction with other antibiotic therapy. **Lysostaphin** will be tested in conjunction with other antibiotics, including cephalothin, vancomycin, ciprofloxacin, ofloxacin, erythromycin, gentamicin, and tobramycin. The effectiveness of. . . strains will be tested in the cornea, for example, Staphylococcus aureus, Serratia, and Pseudomonas. The infections will be treated with **lysostaphin** plus another antibiotic. A test antibiotic will be administered every 30 min from 4 to 9 hr post-infection. Five minutes after each application of test antibiotic, a topical drop of **lysostaphin** (0.3%) will be administered. In these experiments, there will be an untreated group and a group treated with the test antibiotic alone, and a group treated with **lysostaphin** alone (4 corneas per group). All eyes will undergo SLE scoring at 4 and 10 hr post-infection for Staphylococcus, and. . . groups treated with a single antibiotic versus those treated with the antibiotic combination will be compared. It is expected that **lysostaphin** will demonstrate no inhibitory interactions with other drugs and that **lysostaphin** may be combined with other antibiotics for treatment of a broader range of bacteria.

DETD Uptake and retention of **lysostaphin** in the cornea and aqueous humor

DETD The changes in **lysostaphin** concentration in the cornea and aqueous humor will be measured during and after therapy to determine the pharmacokinetics. Normal corneas and corneas infected with Staphylococcus for 4 hr will be topically treated with 0.3% **lysostaphin** every 30 min for 5 hr. Corneas (six per group) will be assayed for **lysostaphin** 30 min after the first application, one hour after the first application, and every hour thereafter for a total of 8 hr. The assay of **lysostaphin** will be performed by four methods: bacterial lysis, bacterial killing, enzyme activity, and ELISA assay. Bacterial lysis assays are spectrophotometric. . . in optical density at 600 nm) according to the procedure of Kline et al., "A colorimetric microtiter plate assay for **lysostaphin** using a hexaglycine substrate," Analytical Biochemistry, vol. 217, pp. 329-331 (1994). Bacterial killing assays will determine the reduction in CFU/ml

caused by the incubation of Staphylococcus for 1 hr with samples containing **lysostaphin**. The enzyme assay and the ELISA assay will be conducted by the procedure of E. Harlow et al., "Antibodies: A. . .

DETD It is expected that **lysostaphin** will penetrate the cornea and accumulate in the aqueous humor at low concentrations. The extensive bacteria killing is probably due to **lysostaphin**'s effectiveness at much lower levels than required for other antibiotics.

DETD Dose effects of **lysostaphin**

DETD When comparing antibiotics in the rabbit keratitis model, a standard 0.3% solution of **lysostaphin** has been applied every 30 min for 5 hr as described in Example 1. To determine the effect of other concentrations, **lysostaphin** will be formulated to its maximal practical concentration (potentially 5%) and then diluted serially to test both the antibiotic effect. . . . and irritating aspects of four concentrations above 0.3% and four concentrations below 0.3% will be tested. A topical application of **lysostaphin** will be applied every 30 min from 4 to 9 hr post-infection. Effectiveness will be measured by reductions in CFU. . . .

DETD It is expected that concentrations up to 5% **lysostaphin** can be obtained and that a repeated topical application of 5% **lysostaphin** could achieve an intra-corneal concentration of 5 .mu.g/ml.

DETD Immune complications of **lysostaphin** therapy

DETD Because **lysostaphin** is a protein, the possibility of an immune response to the molecule must be considered. Two aspects of an immune response will be considered: first, production of antibodies could neutralize the bactericidal activity of **lysostaphin**; and second, an immune response can result in inflammation or tissue damage (an allergic reaction). Normal (uninfected) rabbits will be topically treated with **lysostaphin** every 30 min for 5 hr. This will be repeated every two weeks for a total of five treatments. Immediately. . .

DETD The SLE scoring will determine if any inflammatory or damaging immune reactions appear as a result of repeated **lysostaphin** application. The sera obtained during weeks 2, 4, 6, and 8 will be used to determine if antibodies are produced.. . . control. Antibody will be detected and quantified by an antibody capture ELISA assay. The ability of any antiserum to block **lysostaphin** activity will be tested in a bacterial lysis assay.

DETD It is expected that any antibody production to **lysostaphin** applications will be low and will not interfere with **lysostaphin** activity. It is also expected that **lysostaphin** will not cause any adverse effects. If antibodies are formed and inhibit drug action or induce allergic reactions, then patients. . .

DETD The term "therapeutically effective amount" as used herein for treatment of keratitis refers to an amount of **lysostaphin** sufficient to decrease a subject's ocular Staphylococcus infection to a statistically significant degree or to decrease the symptoms of a Staphylococcus infection to a statistically significant degree. Ordinary persons skilled in the art would recognize that the effect of **lysostaphin** on a human eye infected with Staphylococcus would correlate with the effects seen in the rabbit eye, a common model. . . .

DETD Pharmaceutically acceptable carrier preparations for topical administration of **lysostaphin** include sterile, aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils,. . . suspensions, including saline and buffered media. This would include sodium chloride solution, Ringer's, ionic resins, or fixed oils. The active **lysostaphin** may be mixed with excipients that are pharmaceutically acceptable and are compatible with the **lysostaphin**. Suitable excipients include water,

saline, dextrose, glycerol and ethanol, or combinations thereof. The **lysostaphin** may also be mixed with pharmaceutically acceptable carriers to form an ointment, including hydrophilic petrolatum, petrolatum, white petrolatum, mineral oils,. . .

DETD **Lysostaphin** may also be mixed with other drugs, including antiinflammatory steroids and antibiotics to treat a broader range of bacteria causing. . .

DETD . . . incorporated by reference. Also incorporated by reference is the complete disclosure of an Abstract by J. J. Dajcs et al., " **Lysostaphin** is effective in treating methicillin-resistant and methicillin-sensitive Staphylococcus aureus keratitis," IOVS, vol. 40, p. S262 (1999), to be presented at. . .

CLM What is claimed is:

. . . in the stromal layer of the cornea in a mammal, comprising the topical application of a therapeutically effective amount of **lysostaphin** to the cornea, wherein the **lysostaphin** penetrates through the outer epithelial layer of the cornea to reach the stromal layer.

4. The method of claim 1, wherein the **lysostaphin** is applied in a concentration between about 0.1% w/v and about 5% w/v.

5. The method of claim 1, wherein the **lysostaphin** is applied in a concentration about 0.3% w/v.

6. The method of claim 1, wherein the **lysostaphin** is applied in combination with a pharmaceutically acceptable carrier.

7. The method of claim 1, wherein the **lysostaphin** is applied in combination with another ocular medication.

. . . in the stromal layer of the cornea in a human, comprising the topical application of a therapeutically effective amount of **lysostaphin** to the cornea, wherein the **lysostaphin** penetrates through the outer epithelial layer of the cornea to reach the stromal layer.

11. The method of claim 8, wherein the **lysostaphin** is applied in a concentration between about 0.1% w/v and about 5% w/v.

12. The method of claim 8, wherein the **lysostaphin** is applied in a concentration about 0.3% w/v.

13. The method of claim 8, wherein the **lysostaphin** is applied in combination with a pharmaceutically acceptable carrier.

14. The method of claim 8, wherein the **lysostaphin** is applied in combination with another ocular medication.

L2 ANSWER 14 OF 34 USPATFULL

AN 2000:21545 USPATFULL

TI Method for the treatment of staphylococcal disease

IN Climo, Michael W., Richmond, VA, United States
Archer, Gordon L., Richmond, VA, United States
Goldstein, Beth P., Tarrytown, NY, United States

PA Ambi Inc., Tarrytown, NY, United States (U.S. corporation)

PI US 6028051 20000222

AI US 1998-140732 19980827 (9)

DT Utility

FS Granted

EXNAM Primary Examiner: Weddington, Kevin E.

LREP Long, Aldridge & Norman, LLP, Kelber, Steven B.

CLMN Number of Claims: 17

ECL Exemplary Claim: 1

DRWN No Drawings
LN.CNT 616
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB **Lysostaphin** is an effective antibiotic in the treatment of staphylococcal infection. Large doses of **lysostaphin** or **lysostaphin** analogues are effective in short course, or even one dose administrations, in treating and eradicating staphylococcal infections, including those resistant. . .

SUMM This invention pertains to the administration of **lysostaphin** for the purpose of treatment of staphylococcus infection in mammals, including humans, as well as pharmaceutical preparations used in said. . . bacteremia; and staphylococcal infection of kidneys, lungs, skin, bone, burns, wounds and prosthetic devices. The invention embraces the use of **lysostaphin** broadly, including not only wild type **lysostaphin** but recombinant **lysostaphin**; **lysostaphin** variants with amino acid sequences varying from the published `natural sequence` of the mature peptide (U.S. Pat. No. 4,931,390) due. . .

SUMM **Lysostaphin** is an enzyme, first identified in Staphylococcus simulans (formerly known as S. staphylolyticus), which has antimicrobial activity by virtue of. . . on glycine-containing bridges in the cell wall peptidoglycan of bacteria (Zygmunt, et al., Progr. Drug Res. 16:309-333 (1972)). In vitro, **lysostaphin** is particularly active against Staphylococcus aureus, because the cell wall bridges of this species contain a high proportion of glycine,. . .

SUMM The activity of **lysostaphin** has also been explored in animal infection models. For the purposes of this discussion, the results of intraperitoneal treatment after. . . of 50% of treated mice after single or multiple subcutaneous administrations of a total of approximately 1 mg/kg of a **lysostaphin** preparation (Schuhardt, et al., J. Bacteriol. 88:815-816 (1964); Harrison, et al., Can. J. Microbiol. 13:93-97 (1967)). A total dosage of. . .

SUMM . . . 41:62-68 (1968); Schaffner, et al., Yale J. Biol. Med. 39:230-244 (1967); Harrison, et al., J. Bacteriol. 93:520-524 (1967)). When a **lysostaphin** preparation was administered intravenously within 6 hours after infection, significant reductions in the numbers of bacteria in the kidneys were. . . seen when treatment was withheld for 24 hours or longer, even with dosages of 125 or 250 mg/kg of a **lysostaphin** preparation. The effect of multiple treatments was not studied.

SUMM . . . The Goldberg, et al., experiment was not comparative, and is therefore of limited utility in assessment of the administration of **lysostaphin**. However, high dosages of **lysostaphin** (at least 50 mg/kg/treatment) were only moderately effective, as judged by the health of the dogs and by the extent. . .

SUMM Limited human trials were conducted aimed at eradication of nasal carriage of S. aureus by topical application of **lysostaphin** to the nares (Martin, et al., J. Lab. Clin. Med. 70:1-8 (1967); Martin, et al., J. Lab. Clin. Med. 71:791-797. . .

SUMM The art reports treatment of one very ill human patient with a single dose of parenterally administered **lysostaphin**, followed by an antibiotic, gentamicin, three days later. The patient died, but did exhibit a reduction in bacteremia (Stark, et. . .

SUMM . . . phenomena observed during the course of the animal and human studies, were noted as a great concern. Contamination of the **lysostaphin** preparations with extraneous substances may have been responsible for at least some of these phenomena.

SUMM . . . of desired effectiveness in the studies discussed. This may have been further due to the difficulty in producing and purifying **lysostaphin**.

SUMM The staphylococcal gene for **lysostaphin** has been sequenced and cloned (U.S. Pat. No. 4,931,390). **Lysostaphin** for use as a

laboratory reagent has been produced by fermentation of a non-pathogenic recombinant strain of Bacillus sphaericus, from. . .

SUMM Although this previous art did not teach that **lysostaphin** is highly effective in clearing established infections from various organs in animal models, more recently it has been demonstrated that a regimen of multiple, relatively low, doses of **lysostaphin** was surprisingly effective in curing experimental endocarditis in rabbits caused by methicillin-resistant Staphylococcus aureus (MRSA) or vancomycin intermediate susceptible S.. . . application Ser. No. 09/120,030, filed Jul. 21, 1998; Climo, et al., Antimicrob. Agents Chemother. 42:1355-1360 (1998).) The good tolerability of **lysostaphin** in the rabbit model suggests that a multiple dose regimen of **lysostaphin**, alone or in combination with other antibiotics, may be practicable in treating human disease. However, it remains an object of those of skill in the art to develop the most tolerable and most effective means of using **lysostaphin** to treat human staphylococcal disease.

SUMM Furthermore, it is known that even the safest drugs can have undesired side effects. Although **lysostaphin** has thus far not been shown to have adverse effects in animal models, other protein drugs are known to cause. . .

SUMM . . . will become more apparent through the disclosure set forth below, are achieved by the administration of relatively high dosages of **lysostaphin**, of at least 50, preferably 100, mg/kg. (As used herein, mg/kg refers to milligrams of **lysostaphin** analogue per kilogram of body weight administered in any 24-hours period). These unprecedented high dosages can include "single dose treatments", where effective protection is provided by a single large dose of **lysostaphin**, as well as "short course administration", or "repeated dose administration". In short course administration, the relatively high dosage, which may. . . followed by one or two repeats of that dosage, separated by perhaps at least a day. Thus, a dose of **lysostaphin** of 100 mg/kg or greater on day 1, day 3 and day 5 or other pattern with greater separation between. . .

SUMM The administration of single or short course, relatively high, dosages of **lysostaphin** (50-100 mg/kg or greater) is a dramatically effective therapy for the treatment of staphylococcal infections, particularly infections that are resistant to treatment, and/or typically associated with significant morbidity and mortality. Further, administered in this way, **lysostaphin** is demonstrated to be effective against staphylococcal bacteria that are at least partially resistant to available antimicrobial agents, such as. . .

SUMM The invention further includes combinatorial therapies, calling for a single or short course high dose of **lysostaphin**, which may be administered before or after initiation of other therapies, and may be followed by two or more days. . . or more other antimicrobial agents; this treatment regimen may be repeated by giving one or more additional high dosages of **lysostaphin**, at intervals of two to 10 days, in the presence or absence of continuing therapy with other antimicrobial agents. Particularly preferred antibiotics for administration in concert with **lysostaphin** according to this invention are rifamycins (isolated from microorganisms or synthetically or semi-synthetically produced, such as rifampin) and glycopeptides (a. . .

SUMM The availability of cloned, recombinant and variant **lysostaphin** further expands this invention. Related enzymes have been identified, and can further be used together with, or in place of, **lysostaphin**.

SUMM The cloning and sequencing of the **lysostaphin** gene permits the isolation of variant enzymes that can have properties similar to or different from those of wild type **lysostaphin**. One such altered enzyme, bearing a single amino acid change, has been

characterized and shown to have potent anti-staphylococcal activity. . .

SUMM Recently, another glycylglycine endopeptidase (ALE-1, from Staphylococcus capitis EPK1) has been described. ALE-1 is distinct from **lysostaphin**, although the two enzymes have considerable amino acid homology (Sugai et al., J. Bacteriol. 179:1193-1202(1997)). Another peptidoglycan hydrolase with a lower degree of homology to **lysostaphin**, but which also possesses endopeptidase activity, is zoocin A, produced by Streptococcus zooepidemicus 4881 (Simmonds et al., Applied and Environmental Microbiology 62:4536-4541 (1996); Simmonds et al., Gene 189:255-261(1997)). Other **lysostaphin** analogues, including naturally occurring enzymes of this type, or even chimeric enzymes obtained by fusing the binding domain of one. . .

SUMM **Lysostaphin** Analogue

SUMM Any enzyme, including **lysostaphin** (wild type), any **lysostaphin** mutant or variant, any recombinant, or related enzyme that retains the proteolytic ability, in vitro and in vivo, of proteolytic. . . the process) or by mutation of the structural gene. Mutations may include site-deletion, insertion, domain removal and replacement mutations. The **lysostaphin** analogues contemplated in the instant invention may be recombinantly expressed or otherwise.

SUMM Administration by injection, including **intravenous**, intramuscular, subcutaneous, intraorbital, intraspinal, intraperitoneal and by direct perfusion or delivery to organs or tissues through injection (e.g., intramedullary). Administration. . .

SUMM While studying the tolerability of high dosages of **lysostaphin** in infected rabbits, it was discovered that single, high dosages were surprisingly efficacious in curing infections. This is demonstrated, below,. . .

SUMM . . . may give concern in some, but not other situations (such as emergency or short term situations) suitably pure preparations of **lysostaphin** analogues, obtained by the fermentation of harmless recombinant strains of bacteria, are expected to be less prone to induce immunogenic. . .

SUMM . . . without additional solutes for osmotic balance) for reconstitution with liquids, suitable for parenteral delivery of the active agent, preferably via **intravenous** (i.v.), intramuscular (i.m.), subcutaneous (s.c.), or intraperitoneal (i.p.) routes or intrathecally or by inhalation or by direct instillation into an. . . thus to effect a reduction in bacterial titers in order to cure or to alleviate an infection. Furthermore, the active **lysostaphin** analogue can be coadministered, at the same time or consecutively, with other antimicrobial agents so as to more effectively treat. . .

SUMM Suitable dosages and regimens of **lysostaphin** may vary with the severity of the infection and the sensitivity of the infecting organism. Dosages may range from 50. . .

DETD All experiments were conducted using **lysostaphin** or a variant enzyme produced by fermentation of recombinant B. sphaericus strains engineered to contain the **lysostaphin** gene described by Recsei (U.S. Pat. No. 4,931,390) or a mutant thereof. Specifically, the **lysostaphin** analogues prepared by fermentation of B. sphaericus varies from the published sequence by having as many as 2 fewer or. . . 2 additional amino acids at the N-terminus. In particular, the data herein are largely derived from studies using preparations of **lysostaphin** analogues wherein the majority component is one that lacks the two N-terminal amino acids of the published sequence. However, the. . .

DETD In Vitro Activity of **Lysostaphin** Against VISA

DETD Prior to conducting infection model studies in animals, the minimal inhibitory activity (MIC) of **lysostaphin** for VISA strains, including two U.S. and one Japanese clinical isolate and a laboratory mutant, was determined to be 0.015-0.03. . . Committee for Clinical

Laboratory Standards, Villanova, Pa.), with the addition of 0.1% (wt/vol) bovine serum albumin to prevent adsorption of **lysostaphin** to plastic pipettes and microtiter trays. The MIC of vancomycin for these strains is 8 .mu.g/ml, twice the generally accepted. . .

DETD Efficacy of Single High Doses of **Lysostaphin** Against Experimental S. aureus Endocarditis in Rabbits

DETD . . . animals were randomly assigned to different treatment groups: untreated control; positive control, vancomycin 30 mg/kg twice daily for 3 days; **lysostaphin** 30 mg/kg twice daily for 3 days; **lysostaphin** 100 mg/kg once; **lysostaphin** 250 mg/kg once; **lysostaphin** 500 mg/kg once. Any rabbits whose infection was not confirmed by pre-treatment blood culture were eliminated. In addition, all rabbits. . . by the presence of an aortic vegetation indicative of an ongoing or a previously existing disease state. All treatments were **intravenous**; the single high doses of **lysostaphin** were administered over 30 minutes, using an infusion pump. The state of health of the rabbits was assessed at intervals. From the rabbits treated with a single high dose of **lysostaphin**, blood samples were withdrawn for culture of bacteria during days 1, 2, and 3 (start of treatment is day 1).. . .

DETD . . . MRSA strains in this infection model (U.S. patent application Ser. No. 09/120,030, filed Jul. 21, 1998; Climo, et al., Ibid.). **Lysostaphin** at the same dosage was highly efficacious in reducing the bacterial count in the heart valve vegetations and also in. . . not unexpected, as similar data were generated previously using a laboratory-derived mutant VISA strain. However, the present data confirm that **lysostaphin** is equally active against clinical VISA isolates in the rabbit infection model. (The same is not true for vancomycin, which. . .

DETD As part of the evaluation of the tolerability of **lysostaphin** in mammals, several rabbits infected with VISA strain HP5827 were treated intravenously once with higher dosages of **lysostaphin**, ranging from 100 to 500 mg/kg. Since all of these rabbits tolerated the **lysostaphin**, they were kept and monitored and later sacrificed for evaluation of bacterial counts in the heart valves and kidneys.

DETD . . . per ml). This is a surprising result, because previous experiments with single dosages of 60 mg/kg vancomycin or 15 mg/kg **lysostaphin** produced only a transient drop in bacteremia, with viable cells detected again in the blood by 24 hours after treatment. . .

DETD Furthermore, as shown in Table 1, in 4 rabbits treated with a single dose of 100 mg/kg of **lysostaphin**, at sacrifice (on day 4) the mean bacterial count in the heart valve vegetations was reduced to about the same extent as in rabbits that received three days of twice daily treatment with 30 mg/kg **lysostaphin** (total dose 180 mg/kg) and, significantly, two of the four rabbits had completely sterile valves (less than log.sub.10 =2 bacteria per gram). Additionally, all 4 rabbits treated once with 100 mg/kg **lysostaphin** had no bacteria detectable in the kidneys.

DETD One rabbit each was treated with of the single doses of 250 and 500 mg/kg **lysostaphin**, respectively. Both of these animals had completely sterile heart valve vegetations and kidneys.

DETD TABLE 1

Efficacy of different **lysostaphin** treatment regimens against S. aureus endocarditis in rabbits (VISA strain HP5827)

Mean log.sub.10 CFU/gram .+-. SD*	Number sterile/total animals treated	
heart. . . vegetation		kidney

Untreated control	10.3 .+-. 0.51	7.46 .+-. 0.6	0/11	0/11
Vancomycin 30 mg/kg twice a day	9.66 .+-. 1.1	3.14 .+-. 1.39	0/9	0/9
Lysostaphin 30 mg/kg twice a day	2.03 .+-. 0.06.sup.a	2.09 .+-. 2.2	5/6.sup.a	4/6.sup.a
Lysostaphin 100 mg/kg once on day 1	2.29 .+-. 033.sup.a	.ltoreq.1.0.sup.a	2/4	4/4.sup.a
Lysostaphin 250 mg/kg once on day 1	.ltoreq.2.0	.ltoreq.1.0	1/1	1/1
Lysostaphin 500 mg/kg once on day 1	.ltoreq.2.0	.ltoreq.1.0	1/1	1/1

*SD: standard deviation of the mean
.sup.a p < 0.05 as compared. . .

DETD TABLE 2

Efficacy of **lysostaphin** against S. aureus endocarditis in rabbits (VISA strain MU-50)

Treatment	Mean log.sub.10 CFU/gram of vegetation	no. sterile/total treated
Untreated control	10.4	0/4
Vancomycin 30 mg/kg twice a day	9.8	0/4
Lysostaphin 30 mg/kg twice a day	2.5	1/2

DETD . . . the heart valve vegetations of 4 of the 6 rabbits, treated with single doses of 100, 250 or 500 mg/kg **lysostaphin** is unprecedented. The rapid action of a single high dose of **lysostaphin** in vivo suggests that short or intermittent regimens

of antimicrobial **lysostaphin** enzyme or analogues could greatly improve the outcome in patients with serious staphylococcal infections that require rapid reduction in bacterial. . .

DETD The above data demonstrate the efficacy of **lysostaphin** against S. aureus that are both MRSA (methicillin-resistant) and vancomycin intermediate susceptible (VISA). These organisms are a newly emerging problem.

DETD . . . is accepted as a rigorous test of the ability of antimicrobial agents to cure severe human infections. Previous work with **lysostaphin** in the rabbit endocarditis model demonstrated the efficacy of **lysostaphin** against infections caused by multiply antibiotic-resistant S. aureus, when the **lysostaphin** was administered in traditional multiple dose, multiple day treatment regimens with or without another antibiotic such as vancomycin. Earlier work. . .

DETD The results presented herein demonstrate not only the unexpected effectiveness of a single high dose of **lysostaphin** against S. aureus endocarditis, but show that such efficacy is far superior to that expected for standard treatments. Currently available. . . of infection to other vital organs. The above results indicate that one or a few treatments with high doses of **lysostaphin** analogues, alone or in combination with standard dosage regimens of other agents, have the potential for effectiveness in the treatment of such infections. Furthermore, based on the in vitro activity of **lysostaphin** against staphylococci (U.S. patent application Ser. No. 09/120,030, filed Jul. 21, 1998), and on the fact that very high doses of **lysostaphin** are well tolerated by rabbits, it is to be expected that **lysostaphin** analogues, alone or in combination with other agents, will also be useful against species of staphylococci other than S. aureus. Among the agents suitable for use together with **lysostaphin** are vancomycin and other glycopeptides, rifampin and other rifamycins, and other anti-infective agents that have activity against staphylococci.

DETD **Lysostaphin** analogues may be used not only in the treatment of staphylococcal endocarditis but other potentially lethal staphylococcal diseases, such as. . . type or severity requiring prolonged treatment with currently used antimicrobial agents. The instant invention further extends to the use of **lysostaphin** analogues in treating such infections and diseases when they are caused by staphylococci that are resistant to routinely used antibiotics.

DETD . . . is not limited to the individual species identified nor should the examples be construed as limiting. A wide variety of **lysostaphin** analogues can be used in the practice of this invention, as can combinatorial agents. Such variations, as well as variations. . .

CLM What is claimed is:

1. A method of treating staphylococcal infection in a patient, comprising: administering to said patient a single dose of **lysostaphin** analogue in a dosage of at least 50 mg **lysostaphin**/kg body weight (mg/kg), wherein said administration is not continued, and said infection is reduced, and wherein said infection is one. . .

6. A method of treating staphylococcal infection in a patient, comprising: administering to said patient an effective amount of **lysostaphin** analogue in a dosage of at least 50 mg/kg/day, wherein said administration is continued for a period of 1-5 days,. . .

11. A method of treating staphylococcal infection in a patient, comprising: administering to said patient an amount of **lysostaphin** analogue in a dosage level of at least 50 mg/kg on a first day of treatment, and repeating said administration once or twice, wherein each said repetition is separated by at least one day on which

lysostaphin is not administered, and said infection is reduced, and wherein said infection is one selected from the group consisting of. . .

16. A composition of matter, comprising a single dosage formulation of **lysostaphin** effective in treating staphylococcal infection in a patient wherein said single dosage composition comprises at least 2,200 mg **lysostaphin** analogue, and a pharmaceutically acceptable carrier.

17. The composition of claim 16, wherein said composition comprises, in addition to said **lysostaphin** analogue, an additional antibiotic agent.

L2 ANSWER 15 OF 34 USPATFULL
AN 2000:12584 USPATFULL
TI Inhibitors of regulatory pathways
IN Bao, Ying, Sunnyvale, CA, United States
Boggs, Amy, Menlo Park, CA, United States
Contag, Pamela R., San Jose, CA, United States
Federspiel, Nancy A., Menlo Park, CA, United States
Hebert, Alan, Menlo Park, CA, United States
Hecker, Scott, Los Gatos, CA, United States
Malouin, Francois, Los Gatos, CA, United States
PA Microcide Pharmaceuticals, Inc., Mountain View, CA, United States (U.S. corporation)
PI US 6020121 20000201
AI US 1996-672215 19960625 (8)
PRAI US 1995-4626 19950929 (60)
DT Utility
FS Granted
EXNAM Primary Examiner: Brusca, John S.
LREP Lyon & Lyon LLP
CLMN Number of Claims: 15
ECL Exemplary Claim: 1
DRWN 20 Drawing Figure(s); 19 Drawing Page(s)
LN.CNT 2350
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
SUMM . . . method of giving a dosage of an antibacterial pharmaceutical composition to a mammal where the method is, e.g., topical, oral, **intravenous**, transdermal, intraperitoneal, intramuscular, or intrathecal. The preferred method of administration can vary depending on various factors, e.g., the components of. . .
DRWD . . . grown in presence of compounds 1, 2, or 3 to a similar density (O.D. 600 nm--0.5-0.6). Cells were lysed using **lysostaphin** prior to separation of cell surface proteins by SDS-PAGE. After electrophoretic transfer of proteins from the gel onto a nitrocellulose. . .
DETD . . . of mice intravenously injected with wild type Staphylococci, where 78% produced positive cultures. These experiments were performed 21 days after **intravenous** injection.
DETD . . . and Gilman's: The Pharmacological Basis of Therapeutics, 8th Ed., Pergamon Press. Methods for administration are discussed therein, e.g., for oral, **intravenous**, intraperitoneal, or intramuscular administration, subcutaneous, topically, and others.

L2 ANSWER 19 OF 34 USPATFULL
AN 1999:4620 USPATFULL
TI Composition for treating mastitis and other staphylococcal infections
IN Blackburn, Peter, New York, NY, United States
Polak, June, Brooklyn, NY, United States
PA Ambi Inc., Tarrytown, NY, United States (U.S. corporation)

PI   US 5858962   19990112
AI   US 1993-168687   19931216 (8)
RLI   Continuation of Ser. No. US 1989-440092, filed on 22 Nov 1989, now abandoned which is a continuation of Ser. No. US 1988-188183, filed on 28 Apr 1988, now abandoned which is a continuation-in-part of Ser. No. US 1987-48412, filed on 11 May 1987, now abandoned
DT   Utility
FS   Granted
EXNAM   Primary Examiner: Weddington, Kevin E.
LREP   White & Case L.L.P.
CLMN   Number of Claims: 14
ECL   Exemplary Claim: 1
DRWN   1 Drawing Figure(s); 1 Drawing Page(s)
LN.CNT 733
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB   **Lysostaphin** is used to eliminate and cure staphylococcal infections including the cure of mastitis by intramammary infusion. Administration of from 2 mg to 400 mg of **lysostaphin** to an infected bovine mammary gland eliminates staphylococci, and the reoccurrence common with antibiotic therapy is not observed. Teat-dips containing **lysostaphin**, mutanolysin and lysozyme can be used as a prophylactic. Synergistic enhancement of the killing effect of **lysostaphin** is observed when a mild surfactant or penicillin or both is included in the formulation.

SUMM   This application relates to the use of **lysostaphin** in the treatment and prevention of staphylococcal infection and, in particular, to the treatment and prevention of staphylococcal bovine mastitis.

SUMM   **Lysostaphin** is a bacteriocin secreted by a single known strain of Staphylococcus simulans originally isolated and named Staphylococcus staphylolyticus by Schindler and Schuhardt. The production of **lysostaphin** by S. staphylolyticus has been described previously in U.S. Pat. No. 3,278,378 issued Oct. 11, 1966 and in Proceedings of the National Academy of Sciences, Vol. 51, pp. 414-421 (1964). The single organism S. staphylolyticus (NRRL B-2628) which produced **lysostaphin** was recently identified as a biovar of S. simulans by Sloan et al., Int. J. System. Bacteriol., Vol. 32, pp. 170-174 (1982). Since the name S. staphylolyticus is not on the Approved List of Bacterial Names, the organism producing **lysostaphin** has been redesignated as S. simulans.

SUMM   Bacteriocins are proteins secreted by bacteria that kill and sometimes lyse related bacteria. For example, **lysostaphin** lyses and kills practically all known staphylococcal species but is inactive against bacteria of all other genera. **Lysostaphin**, isolated from culture filtrates of S. simulans (NRRL B-2628) grown according to published references, is an endopeptidase which cleaves the polyglycine cross-links of the peptidoglycan found in the cell walls of staphylococci. In addition, cultures that produce **lysostaphin** appear to be resistant to its activities while cultures grown under non-**lysostaphin** producing conditions are sensitive.

SUMM   Previous studies have shown that **lysostaphin** can be produced by fermentation techniques wherein S. simulans is grown in liquid culture. Such fermentation techniques are described in. . . and in Proceedings of the National Academy of Sciences, Vol. 51, pp. 414-421 (1964). Various improvements in the production of **lysostaphin** by fermentation techniques have also been made as documented in U.S. Pat. Nos. 3,398,056, issued Aug. 20, 1968, and 3,594,284,. . . issued Jul. 20, 1971. The latter two references disclose improvements to culture medium and inoculation techniques whereby the production of **lysostaphin** by fermentation can be accelerated and improved. **Lysostaphin** is produced by S. simulans during exponential growth as an inactive precursor. The proenzyme is converted to active mature enzyme. . .

SUMM In addition, **lysostaphin** can be produced by recombinant microorganisms, including strains of E. coli, Bacillus subtilis and B. sphaericus which express the **lysostaphin** gene. In contrast to the natural production, **lysostaphin** accumulates during exponential growth in the culture medium of recombinant **lysostaphin** producing strains as fully processed mature active enzyme and is free of staphylococcal immunogenic contaminants.

SUMM Studies on the possible mechanism of antibiotic evasion of phagocytized staphylococci in mastitis treatment show that **lysostaphin** had been rejected as a candidate for destroying phagocytized staphylococci. Craven et al., 29 Research in Veterinary Science 57 (1980);. . . Comp. Immun. Microbial. Infect. Dis. 447 (1982)) Craven et al., 51 Journal of Dairy Research 513 (1984). In these experiments **lysostaphin** was used in vitro as a pretreatment to destroy extracellular staphylococci prior to exposing the phagocytized staphylococci to cloxacillin, gentamicin or **lysostaphin**. Craven et al.'s results strongly suggest that **lysostaphin** would have no effect on mastitis since intracellular staphylococci were still viable after 20 hours of incubation in a **lysostaphin** containing solution. 51 Journal of Dairy Research at 515-516, and Table 2.

SUMM **Lysostaphin** has also been reported to penetrate human monocytes. Since monocytes are a different cell type than PMNs, this human model. . .

SUMM **Lysostaphin** has also been shown to be effective in the treatment of staphylococcal renal abscesses in mice, particularly when used in. . .

SUMM In man **lysostaphin** has also been used as a therapeutic agent for treatment of chronic nasal staphylococcal infections (Quickel, Jr. et al., 22 Applied Microbiology 446 (1971)). In one case of a resistant staphylococcal infection, **lysostaphin** was given systemically (Stark et al., 291 Medical Intelligence 239 (1974)). In general, however, there has been great skepticism and reluctance in the medical and veterinary communities concerning the **systemic** administration of **lysostaphin**. **Lysostaphin** was considered to be too highly immunogenic to have general use for anything but topical applications.

SUMM It has now been found that **lysostaphin** can be used with surprising effectiveness to prevent and/or cure staphylococcal mastitis, even in its chronic form, without any adverse immunogenic effects. As a prophylactic, **lysostaphin** can be introduced as part of a daily teat-dipping regimen. **Lysostaphin** can be used alone but preferably, the teat-dip will include **lysostaphin**; other bacteriolytic agents such as mutanolysin, a bacteriocin produced by Streptococcus globisporus which is effective against streptococci; and lysozyme, a. . .

SUMM . . . components of the teat dip can be infused into the infected udder to eliminate the bacteria and cure mastitis, e.g., **lysostaphin** alone or with a mild surfactant which surprisingly potentiates the staphylocidal effect of **lysostaphin** more than 1000 times. Furthermore, the combination of **lysostaphin** and penicillin also exhibits synergy such that a 1000 fold increase in the killing of staphylococci is observed in vitro.. . .

SUMM Infusions of a therapeutally effective amount of **lysostaphin**, with or without surfactant, EDTA, penicillin or other potentiating agents, are used to achieve elimination of the staphylococcal infection. Preferably such infusions contain between 2 to 400 mg **lysostaphin** when no potentiating agents are present. In combinations containing potentiating agents, the required effective doses of **lysostaphin** can be lowered (as a result of its synergistically enhanced activity) by as much as 1000-fold.

SUMM Synergistic bactericidal activity of **lysostaphin** and

penicillin was observed even upon administration to penicillinase-positive S. aureus and methicillin-resistant S. aureus ("MRSA"). MRSA are usually resistant to multiple antibiotics and are particularly problematic, especially in humans, as well as difficult to kill. The **lysostaphin**/penicillin combination would be indicated for use in specific situations where grave MRSA infection cannot be controlled by conventional antibiotic (e.g. penicillin) therapy. In addition, penicillin and other similar acting substances may also be useful together with **lysostaphin** as an agent against staphylococcal infection and contamination.

DRWD FIG. 1 shows a chromatogram of **lysostaphin** produced by transformant B. sphaericus strain 00 containing the recombinant plasmid pBC16-1L which codes for **lysostaphin**.

DETD **Lysostaphin** for use according to the claimed invention can be obtained from either natural or recombinant sources. Preferably, the **lysostaphin** is obtained from Bacillus sphaericus strain 00 containing a recombinant plasmid which directs the synthesis of **lysostaphin**, as this provides for both high levels of **lysostaphin** production substantially free from staphylococcal immunogenic contaminants and facile **lysostaphin** purification since the **lysostaphin** accumulates directly in the growth medium. Bacillus sphaericus transformants containing the plasmid pBC16-1L have been found to be particularly suited for this purpose, although other strains are also useful as a source of **lysostaphin**. One method for obtaining **lysostaphin** from micro-organisms transformed by recombinant plasmids containing the gene which codes for **lysostaphin** is fully disclosed in U.S. patent application Ser. No. 034,464, filed Apr. 10, 1987, which is a continuation-in-part of U.S.. . . .

DETD Prophylactic treatments for bovine mastitis according to the invention involve the use of **lysostaphin**-containing teat dips. **Lysostaphin**-containing teat dips provide effective prevention of bovine mastitis when used before and after every milking. Preferably, the preventative regimen is used for all cows in the herd. The teat dips comprise about 1.0 .mu.g/ml **lysostaphin** in an acceptable carrier. In addition, teat dips for use according to the invention may include about 1.0 .mu.g/ml mutanolysin,. . .

DETD Intramammary infusion of **lysostaphin** can be used to effectively treat infected animals who have developed either chronic or acute staphylococcal bovine mastitis despite prophylactic treatment. A single dose of from 2 to 400 mg **lysostaphin** per milk gland will eliminate the infection and cure staphylococcal mastitis in most instances. Additional doses of **lysostaphin** may be indicated where the infection is persistent. Doses significantly higher than 400 mg are not recommended as they can. . . In life-threatening cases, the route of administration could also include sites other than the infected gland so as to achieve **systemic** delivery, i.e., **intravenous**, subcutaneous, or intramuscular, and rectal or oral administration of suitably encapsulated formulations in which the **lysostaphin** is protected from inactivation in the gut.

DETD It has also been found that infusion of a combination of **lysostaphin** and penicillin is surprisingly much more efficacious than **lysostaphin** alone because of an apparent synergistically enhanced bactericidal activity of this combination. In addition, it is believed that the therapeutic **lysostaphin** formulation may also include other agents which potentiate the bactericidal activity of **lysostaphin**, for example, synthetic penicillins and other antibiotics, chelating agents, mild surfactants, (e.g., deoxycholate) and other membrane active agents which may facilitate penetration of **lysostaphin** to the site of infection. In formulations that include e.g., penicillin, the dosage of **lysostaphin** can be decreased as a result of the potentiated bactericidal activity of

lysostaphin. Since too high a dose of **lysostaphin** can induce unwanted and potentially adverse side effects, this synergistic effect is significant not only for efficacy but also for. . .

DETD In vitro experiments were conducted to determine the bactericidal activity of **lysostaphin**, mutanolysin, and lysozyme compositions toward S. aureus and other mastitis pathogens. The protocol was as follows:

DETD . . . suspension and 1 ml of control and teat dip test formulation (i.e. milk, buffer, or buffered detergent etc., containing the **lysostaphin** composition) were combined.

DETD The results of in vitro experiments demonstrating the bactericidal efficacy of various **lysostaphin** therapeutic formulations are presented in Tables IA-IC. The results are presented as the percent survivals for S. aureus strains Newbould. . .

DETD Table IA presents results for formulations containing 1 .mu.g/ml, 0.1 .mu.g/ml, 0.01 .mu.g/ml and 0.00 .mu.g/ml (CNTRL) **lysostaphin**. As can be seen from these results all levels of **lysostaphin** tested were effective to kill the organisms in a buffer vehicle (50 mM Tris, pH 8.0). In a milk vehicle,. . .

DETD Table IB shows the effect of adding a mild nonionic surfactant, octylphenoxyl polyethoxy (10) ethanol, (Triton X-100), to the **lysostaphin** formulation. For example, less than 0.001% of the cells survive exposure to 0.1 .mu.g/ml **lysostaphin** and 0.1% Triton X-100, while 2.2% and 7.7%, respectively, survived exposure to each compound alone. Even more surprising, less than 0.001% survival was observed for 0.01 .mu.g/ml **lysostaphin** and 0.1% Triton X-100.

DETD Table IC demonstrates the synergistic effect of **lysostaphin** /penicillin combinations on three strains of staphylococci. Depending on the doses of each, the combinations of **lysostaphin** plus penicillin can be 100 to 1000 times more effective than either **lysostaphin** or penicillin alone with all three strains.

DETD Table ID demonstrates the effect of the combination of **lysostaphin** and penicillin compared with their sequential effect on S. aureus. S. aureus were suspended at 10.sup.7 cells/ml in milk and incubated for the times indicated in the table with either **lysostaphin** and penicillin together or sequentially. After incubation, samples were centrifuged to obtain cell pellets which were washed twice, resuspended in. . . forming units (CFU) were scored after incubation overnight at 37.degree. C. to determine percent survival relative to appropriate controls. The **lysostaphin** /penicillin combination, exhibits a synergistically enhanced bactericidal activity against S. aureus which is at least 3 orders of magnitude greater than. . .

DETD TABLE IA

The Effect of **Lysostaphin** On The Viability of S. Aureus

Strain	Vehicle	Incubation Time	% Survival 1.0L	0.1L	0.01L	CNTRL
S. aureus						
	Milk	15'	2.8	75.0	100. . .	

DETD TABLE IB

The Effect Of Non-Ionic Detergent On The Bactericidal Activity of **Lysostaphin** Toward S. aureus

Incubation	% Survival 0.1L +	0.01L +

Strain
Vehicle
Time
0.1L
0.01L
0.1% T
0.1% T
0.1%. . .

DETD TABLE IC

The Effect of Penicillin On The Bactericidal Activity of **Lysostaphin** Toward S. aureus

		Incuba-	% Survival					
		tion			0.1L +	0.01L +		
Strain	Vehicle	Time	0.1L	0.01L	0.1P	0.1P	0.1P	CNTRL
S. aureus								

. . .

DETD TABLE ID

A Comparison of the Effect of the Combination of **Lysostaphin** and Penicillin Versus Their Sequential Effects on the Survival of Staphylococcus aureus (Strain RN451) in milk at 37.degree. C.

	combo(2h)	lspr(2h)	pen(2h)	Pen(2h)/ lspn(0.5h)	lspn(2h)/ pen(0.5h)
% survival	0.0005	23	25	0.3	10

lspn = **lysostaphin**; pen = penicillin

DETD In addition, assays for **lysostaphin**, mutanolysin, and lysozyme activities which measure the decrease in turbidity at 600 nm of suspensions of live S. aureus, S.. . .

DETD The data indicate that **lysostaphin** is a rapidly acting, highly effective staphylocide, the bactericidal activity of which is potentiated more than 1000 times by penicillin or the mild surfactant, Triton X-100. The inclusion of a chelating agent further potentiates the bactericidal activity of **lysostaphin**. It is also believed that synthetic penicillins and cell wall-active antibiotics will potentiate the activity of **lysostaphin**. **Lysostaphin** is an effective staphylocide in milk, but in buffer the bactericidal activity of **lysostaphin** is approximately 10 times that observed in milk.

DETD . . . the general protocol described in Examples 1-4, further in vitro experiments were performed to evaluate the bactericidal activity of a **lysostaphin** composition comprising bacteriolytic enzymes, a non-ionic detergent and buffered chelating agent. As shown in Table II

a formulation containing 1% Triton X-100, 0.1 .mu.g/ml **lysostaphin**, 10 .mu.g/ml lysozyme, and 5 mM EDTA in 20 mM Tris, pH 8.0, (AMBI Teat Dip-0.1) was extremely effective against. . .

DETD Trials on cows were performed which demonstrated the efficacy of **lysostaphin** teat-dip compositions in vivo. The tests were performed generally according to Protocol A of the National Mastitis Council. In general,. . . and allowed to air dry for 30 minutes. Two teats (right fore and left rear) were then dipped in a **lysostaphin** test teat dip formulation (10 .mu.g/ml **lysostaphin** in 0.85% saline) to cover 2/3 of the teat, and allowed to air dry for 30 minutes; the remaining two. . .

DETD Ten .mu.g/ml solutions of **lysostaphin** in 0.85% saline completely disinfected invading S. aureus from cow teat surfaces. Moreover, **lysostaphin** applied to teat surfaces prior to exposure of teats to S. aureus suspensions had sufficient residual activity on the teat. . . of the teat. Residual activity could be enhanced by inclusion of a polymeric adsorbent and/or inert carrier protein to reduce **lysostaphin** wash-off.

DETD . . . Example 6 and the data obtained in vitro, an enhanced teat dip formulation (AMBI Teat Dip 1.0) comprising 1.0 .mu.g/ml **lysostaphin**, 10 .mu.g/ml lysozyme, 1.0% Triton X-100, and 5 mM EDTA in 20 mM Tris buffer, pH 8.0 was evaluated as. . . to air dry for 30 min. The treated teats were then dipped in AMBI test teat dip-1.0 solution (1.0 .mu.g/ml **lysostaphin**, 10.0 .mu.g/ml lysozyme, 1.0% Triton X-100, 5 mM EDTA, 20 mM Tris buffer, pH 8.0) and allowed to air dry. . .

DETD . . . 200-300 CFU of S. aureus strain Newbould 305. Three days post-infection, the glands were infused with a single dose of **lysostaphin** dissolved in 200 .mu.1 0.85% sterile saline. Milk samples were collected from the glands 6 hours after treatment and at. . . were plated on blood agar. After 24-48 hours incubation, the plates were counted to determine CFU. The single doses of **lysostaphin** which were sufficient to eliminate the infection did not produce adverse side effects and indicated that intramammary infusions of **lysostaphin** are effective against staphylococcal mastitis. At 125 .mu.g/kg, glands were cleared of infection by the 6 hour post-treatment sample and. . .

DETD TABLE IV

Efficacy of Intramammary Infusion of **Lysostaphin** Toward Experimental STAPHYLOCOCCAL Mastitis in Guinea Pig

	Lysostaphin Dose .mu.g/kg					
	ZERO	1.0	5.0	25.0	62.5	125.0
Number of animals cleared of infection	(0/10)	(1/0)	(1/2)	(2/2)	(1/1)	(7/7)

DETD It can be seen from these examples that **lysostaphin** is effective for treatment of staphylococcal mastitis and that its effect is greatly enhanced when used in combination with penicillin. . .

DETD Production of **Lysostaphin** from Bacillus

DETD **Lysostaphin** for use according to the claimed invention can be obtained from either natural or recombinant sources. Preferably, the **lysostaphin** is obtained from cultures derived from Bacillus sphaericus strain 00 transformed by recombinant plasmids which direct **lysostaphin** synthesis as described in copending application Ser. No. 034,464 filed Apr. 10, 1987 which is a continuation-in-part of Ser.

No. 852,407 filed Apr. 16, 1986. This method provides for both high levels of **lysostaphin** production substantially free from staphyloccal immunogenic contaminants. **Lysostaphin** purification is facilitated since active **lysostaphin** accumulates directly in the growth medium. Using this method, Bacillus sphaericus 00 transformants containing plasmid pBC16-1L (B. sphaericus 00/pBC16-1L) have. . . found to be particularly suited for the purpose, although other transformed Bacillus strains are also useful as a source of **lysostaphin**.

DETD The **lysostaphin**-producing organism is grown under conditions conducive to the production of **lysostaphin**. The optimum conditions will vary from strain to strain; however, certain types of growth media and fermentation conditions are known to enhance **lysostaphin** production. In the case of the Bacillus sphaericus 00/pBC16-1L transformant, the preferred growth medium is VY broth (25 g Veal. . .

DETD TABLE V

Effect of Aeration on **Lysostaphin** Production
by the Bacillus Spaericus 00/pBC 16-1L Transformant

	Stirring Speed			
Klett	100 rpm	200 rpm	200 rpm (Fluted)	320 rpm
250	21.8	36.2. . .		

culture. Growth medium: VY broth containing 5 .mu.g/ml erythromycin.
Samples were removed at times throughout growth. Supernatants were assaye for **lysostaphin** activity by turbidometric clearing of dead cell suspensions of S. aureus. Results are presented as .mu.g **lysostaphin** per ml.

DETD B. sphaericus 00/pBC16-1L transformant grown on VY medium produced and secreted approximately 130 mg **lysostaphin** per liter of culture medium, which is more than four times the amount produced by S. simulans under the best fermentation conditions currently available. **Lysostaphin** accumulates in the growth medium with little or no degradation, even after prolonged incubation of cultures, and accounts for more. . .

DETD **Lysostaphin** is isolated from the growth medium in accordance with known fractional precipitation (salting out) procedures. Alternatively, a particularly effective purification is achieved by combining a precipitation and a chromatographic separation of the fermentation broth from cultures of the **lysostaphin**-producing B. sphaericus 00/pBC16-1L transformant.

DETD . . . ammonium sulfate is added to the supernatant to 40-60%, preferably 50%, of saturation. After 1 hour at 4.degree. C., the **lysostaphin**-containing precipitate is recovered by centrifugation. Recovery at this step is greater than 80%.

DETD . . . FPLC Mono S) and eluted using a buffered gradient of increasing salt concentration from 0.05 to 0.25M NaCl. Recovery of **lysostaphin** for the single chromatographic step was more than 90%. **Lysostaphin** activity is associated with two major peaks (FIG. 1). The later eluting peak of **lysostaphin** is comprised of non-covalent aggregates of the protein. These aggregates dissociate on dilution in buffer and under conditions of sodium. . .

DETD Construction of the plasmid vector pBC16-1L which contains the gene coding for **lysostaphin** **Lysostaphin**-producing strains of Bacillus sphaericus can be produced using recombinant DNA techniques and preferably those described in copending application Ser. Nos.. . . gene (i.e. .beta.-galactosidase gene). The ligation mix is then

transferred to E. coli (JM105) by transformation. Successful insertions of the **lysostaphin** gene into the plasmid can be found by selecting for transformants by growth on the appropriate antibiotic, and then finding those with a lac Z' negative phenotype. **Lysostaphin** production is detected by turbidometric clearing of a suspension of S. aureus either in solution format or as an overlay. . . .

DETD Using various **lysostaphin**-producing E. coli JM105 transformants, restriction analysis and subcloning of the JM105 plasmid DNA showed that the DNA sequence coding for **lysostaphin** was localized to a 1.5 kbp Hpa II-Hind III DNA fragment. This fragment was visualized after electrophoresis by ethidium bromide. . . . Tris, 1 mM EDTA, pH 8.0). Recombinant plasmids capable of transforming B. subtilis as well as B. sphaericus to express **lysostaphin** were constructed using a derivative of plasmid pBC16 (pBC16-1) as a cloning vector. pBC16 is a tetracycline resistant (Tet.sup.r) Bacillus. . .

DETD . . . The Pvu II-digested vector pBC16-1 was treated with calf intestinal alkaline phosphatase. The 1.5 Kbp DNA fragment which codes for **lysostaphin** was treated with the Klenow fragment of DNA polymerase. The 1.5 Kbp DNA fragment and plasmid DNA were then mixed. . . ligation mixture was transferred to B. subtilis by protoplast transformation. Transformants were resistant to erythromycin, sensitive to tetracycline, and produced **lysostaphin** as indicated by zones of clearing when grown on agar containing dead S. aureus cells. One such **lysostaphin** producing clone was picked and designated B. subtilis/pBC16-1L. Plasmid pBC16-1L DNA extracted from the B. subtilis/pBC16-1L transformant was isolated after. . . by protoplast transformation to various species of Bacillus, including B. sphaericus strain 00. Transformants were resistant to erythromycin and produced **lysostaphin**. The B. sphaericus 00/pBC16-1L transformant provides maximum production of **lysostaphin** and permit accumulation of intact, enzymically active product. B. sphaericus strain 00 was originally isolated from soil and is maintained. . .

CLM What is claimed is:

1. A composition for killing staphylococci comprising **lysostaphin** and an agent which synergistically enhances the bactericidal activity of the **lysostaphin**, and which is in an amount effective to produce the synergistic enhancement, selected from the group consisting of penicillin, bacitracin, methicillin, cephalosporin and polymyxin and wherein the **lysostaphin** and the agent are together in amounts effective to kill staphylococci.

2. A composition for killing staphylococci comprising **lysostaphin** and at least one agent which synergistically enhances the bactericidal activity of the **lysostaphin**, and which is in an amount effective to produce the synergistic enhancement, selected from the group consisting of chelating agents and mild surfactants and wherein both the **lysostaphin** and the agent(s) are together in amounts effective to kill staphylococci.

. . . . 3. A composition according to claim 1 which further comprises at least one agent which synergistically enhances bactericidal activity of **lysostaphin** selected from the group consisting of chelating agents and mild surfactants.

4. A composition according to claim 1, 2 or 3 wherein the **lysostaphin** is present at a concentration of at least 0.01 .mu.g/ml.

. . . A composition according to claim 1 or 3, containing penicillin in an amount effective to potentiate the killing effect of **lysostaphin** .

. . . to claim 2 or 3, containing a mild surfactant in an amount effective to potentiate the killing effect of the **lysostaphin**.

. . . according to claim 3, containing penicillin an a mild surfactant in amounts effective to potentiate the killing effect of the **lysostaphin**.

13. A composition according to claim 1, 2 or 3, wherein the **lysostaphin** is derived from a transformant microorganism containing a recombinant plasmid which codes for **lysostaphin**.

L2 ANSWER 21 OF 34 USPATFULL
AN 1998:108381 USPATFULL
TI Compositions with activity against helicobacter
IN Blackburn, Peter, New York, NY, United States
Goldstein, Beth P., Tarrytown, NY, United States
Cook, Debra J., New York, NY, United States
PA AMBI Inc., Tarrytown, NY, United States (U.S. corporation)
PI US 5804549 19980908
AI US 1996-770521 19961220 (8)
PRAI US 1996-9872 19960105 (60)
DT Utility
FS Granted
EXNAM Primary Examiner: Weddington, Kevin E.
LREP White & Case L.L.P.
CLMN Number of Claims: 11
ECL Exemplary Claim: 1
DRWN 1 Drawing Figure(s); 1 Drawing Page(s)
LN.CNT 510
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
SUMM . . . the Gram-positive bacterial species Streptococcus agalactiae and Listeria monocytogenes. U.S. Pat. No. 4,980,163 discloses that a monoglyceride in combination with **lysostaphin**, a lanthionine-containing bacteriocin such as nisin and the chelating agent EDTA, enhances the bactericidal activity of the composition against Staphylococcus. . .
DETD . . . be expected to have fewer side effects than those associated with agents such as antibiotics that are absorbed into the **systemic** circulation, or that pass into the intestine. Such antibiotics may adversely affect the normal intestinal microflora, thereby, enabling opportunistic pathogens. . .

L2 ANSWER 24 OF 34 USPATFULL
AN 1998:61641 USPATFULL
TI Method for treating mastitis and other staphylococcal infections
IN Blackburn, Peter, New York, NY, United States
Polak, June, Brooklyn, NY, United States
PA Ambi Inc., Tarrytown, NY, United States (U.S. corporation)
PI US 5760026 19980602
AI US 1994-303551 19940909 (8)
RLI Continuation of Ser. No. US 1992-935121, filed on 20 Aug 1992, now abandoned which is a continuation of Ser. No. US 1990-535286, filed on 8 Jun 1990, now abandoned which is a continuation of Ser. No. US 1988-188183, filed on 28 Apr 1988, now abandoned which is a continuation-in-part of Ser. No. US 1987-48412, filed on 11 May 1987, now abandoned
DT Utility
FS Granted
EXNAM Primary Examiner: Weddington, Kevin E.
LREP White & Case L.L.P.
CLMN Number of Claims: 5

ECL Exemplary Claim: 1
DRWN 1 Drawing Figure(s); 1 Drawing Page(s)
LN.CNT 844
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB **Lysostaphin** is used to eliminate and cure staphylococcal infections including the cure of mastitis by intramammary infusion. Administration of from 2 mg to 400 mg of **lysostaphin** to an infected bovine mammary gland eliminates staphylococci, and the reoccurrence common with antibiotic therapy is not observed. Teat-dips containing **lysostaphin**, mutanolysin and lysozyme can be used as a prophylactic. Synergistic enhancement of the killing effect of **lysostaphin** is observed when a mild surfactant or penicillin or both is included in the formulation.

SUMM This application relates to the use of **lysostaphin** in the treatment and prevention of staphylococcal infection and, in particular, to the treatment and prevention of staphylococcal bovine mastitis.

SUMM **Lysostaphin** is a bacteriocin secreted by a single known strain of Staphylococcus simulans originally isolated and named Staphylococcus staphylolyticus by Schindler and Schuhardt. The production of **lysostaphin** by S. staphylolyticus has been described previously in U.S. Pat. No. 3,278,378 issued Oct. 11, 1966 and in Proceedings of the National Academy or Sciences, Vol. 51, pp. 414-421 (1964). The single organism S. staphylolyticus (NRRL B-2628) which produced **lysostaphin** was recently identified as a biovar of S. simulans by Sloan et al., Int. J. System. Bacteriol., Vol. 32, pp. 170-174 (1982). Since the name S. staphylolyticus, is not on the Approved List of Bacterial Names, the organism producing **lysostaphin** has been redesignated as S. simulans.

SUMM Bacteriocins are proteins secreted by bacteria that kill and sometimes lyse related bacteria. For example, **lysostaphin** lyses and kills practically all known staphylococcal species but is inactive against bacteria of all other genera. **Lysostaphin**, isolated from culture filtrates of S. simulans (NRRL B-2628) grown according to published references, is an endopeptidase which cleaves the polyglycine cross-links of the peptidoglycan found in the cell walls of staphylococci. In addition, cultures that produce **lysostaphin** appear to be resistant to its activities while cultures grown under non-**lysostaphin** producing conditions are sensitive.

SUMM Previous studies have shown that **lysostaphin** can be produced by fermentation techniques wherein S. simulans is grown in liquid culture. Such fermentation techniques are described in. . . and in Proceedings of the National Academy of Sciences, Vol. 51, pp. 414-421 (1964). Various improvements in the production of **lysostaphin** by fermentation techniques have also been made as documented in U.S. Pat. Nos. 3,398,056, issued Aug. 20, 1968, and 3,594,284,. . . issued Jul. 20, 1971. The latter two references disclose improvements to culture medium and inoculation techniques whereby the production of **lysostaphin** by fermentation can be accelerated and improved. **Lysostaphin** is produced by S. simulans during exponential growth as an inactive precursor. The proenzyme is converted to active mature enzyme. . .

SUMM In addition, **lysostaphin** can be produced by recombinant microorganisms, including strains of E. coli, Bacillus subtilis and B. sphaericus which express the **lysostaphin** gene. In contrast to the natural production, **lysostaphin** accumulates during exponential growth in the culture medium of recombinant **lysostaphin** producing strains as fully processed mature active enzyme and is free of staphylococcal immunogenic contaminants.

SUMM Studies on the possible mechanism of antibiotic evasion of phagocytized staphylococci in mastitis treatment show that **lysostaphin** had been rejected as a candidate for destroying phagocytized staphylococci. Craven et al., 29 Research in Veterinary Science 57 (1980);. . .

Comp. Immun. Microbial. Infect. Dis. 447 (1982)) Craven et al., 51 Journal of Dairy Research 513 (1984). In these experiments **lysostaphin** was used in vitro as a pretreatment to destroy extracellular staphylococci prior to exposing the phagocytized staphylococci to cloxacillin, gentamicin or **lysostaphin**. Craven et al.'s results strongly suggest that **lysostaphin** would have no effect on mastitis since intracellular staphylococci were still viable after 20 hours of incubation in a **lysostaphin** containing solution. 51 Journal of Dairy Research at 515-516, and Table 2.

SUMM **Lysostaphin** has also been reported to penetrate human monocytes. Since monocytes are a different cell type than PMNs, this human model. . . to be applicable to the treatment of bovine mastitis (van den Broek et al., 21 Scand. J. Immunol 189 (1985)) **Lysostaphin** has also been shown to be effective in the treatment of staphylococcal renal abscesses in mice, particularly when used in. . .

SUMM In man **lysostaphin** has also been used as a therapeutic agent for treatment of chronic nasal staphylococcal infections (Quickel, Jr. et al., 22 Applied Microbiology 446 (1971)). In one case of a resistant staphylococcal infection, **lysostaphin** was given systemically (Stark et al., 291 Medical Intelligence 239 (1974)). In general, however, there has been great skepticism and reluctance in the medical and veterinary communities concerning the **systemic** administration of **lysostaphin**. **Lysostaphin** was considered to be too highly immunogenic to have general use for anything but topical applications.

SUMM It has now been found that **lysostaphin** can be used with surprising effectiveness to prevent and/or cure staphylococcal mastitis, even in its chronic form, without any adverse immunogenic effects. As a prophylactic, **lysostaphin** can be introduced as part of a daily teat-dipping regimen. **Lysostaphin** can be used alone but preferably, the teat-dip will include **lysostaphin**; other bacteriolytic agents such as mutanolysin, a bacteriocin produced by Streptococcus globisporus which is effective against streptococci; and lysozyme, a. . .

SUMM . . . components of the teat dip can be infused into the infected udder to eliminate the bacteria and cure mastitis, e.g., **lysostaphin** alone or with a mild surfactant which surprisingly potentiates the staphylocidal effect of **lysostaphin** more than 1000 times. Furthermore, the combination of **lysostaphin** and penicillin also exhibits synergy such that a 1000 fold increase in the killing of staphylococci is observed in vitro.. . .

SUMM Infusions of a therapeutally effective amount of **lysostaphin**, with or without surfactant, EDTA, penicillin or other potentiating agents, are used to achieve elimination of the staphylococcal infection. Preferably such infusions contain between 2 to 400mg **lysostaphin** when no potentiating agents are present. In combinations containing potentiating agents, the required effective doses of **lysostaphin** can be lowered (as a result of its synergistically enhanced activity) by as much as 1000-fold.

SUMM Synergistic bactericidal activity of **lysostaphin** and penicillin was observed even upon administration to penicillinase-positive S. aureus and methicillin-resistant S. aureus ("MRSA"). MRSA are usually resistant to multiple antibiotics and are particularly problematic, especially in humans, as well as difficult to kill. The **lysostaphin**/penicillin combination would be indicated for use in specific situations where grave MRSA infection cannot be controlled by conventional antibiotic (e.g. penicillin) therapy. In addition, penicillin and other similar acting substances may also be useful together with **lysostaphin** as an agent against staphylococcal infection and contamination.

SUMM While the utility of the **lysostaphin** containing formulations according to the invention is illustrated using mastitis treatment, the enhanced effectiveness of the **lysostaphin** in these formulations makes them suitable for a number of other applications involving staphylococcal infection and contamination. Thus, the formulations. . .

DRWD FIG. 1 shows a chromatogram of **lysostaphin** produced by transformant B. sphaericus strain 00 containing the recombinant plasmid pBC16-1L which codes for **lysostaphin**.

DETD **Lysostaphin** for use according to the claimed invention can be obtained from either natural or recombinant sources. Preferably, the **lysostaphin** is obtained from Bacillus sphaericus strain 00 containing a recombinant plasmid which directs the synthesis of **lysostaphin**, as this provides for both high levels of **lysostaphin** production substantially free from staphylococcal immunogenic contaminants and facile **lysostaphin** purification since the **lysostaphin** accumulates directly in the growth medium. Bacillus sphaericus transformants containing the plasmid pBC16-1L have been found to be particularly suited for this purpose, although other strains are also useful as a source of **lysostaphin**. One method for obtaining **lysostaphin** from micro-organisms transformed by recombinant plasmids containing the gene which codes for **lysostaphin** is fully disclosed in U.S. patent application 034,464, filed Apr. 10, 1987, which is a continuation-in-part of U.S. application 852,407.. . .

DETD Prophylactic treatments for bovine mastitis according to the invention involve the use of **lysostaphin**-containing teat dips. **Lysostaphin**-containing teat dips provide effective prevention of bovine mastitis when used before and after every milking. Preferably, the preventative regimen is used for all cows in the herd. The teat dips comprise about 1.0 .mu.g/ml **lysostaphin** in an acceptable carrier. In addition, teat dips for use according to the invention may include about 1.0 .mu.g/ml mutanolysin,. . .

DETD Intramammary infusion of **lysostaphin** can be used to effectively treat infected animals who have developed either chronic or acute staphylococcal bovine mastitis despite prophylactic treatment. A single dose of from 2 to 400 mg **lysostaphin** per milk gland will eliminate the infection and cure staphylococcal mastitis in most instances. Additional doses of **lysostaphin** may be indicated where the infection is persistent. Doses significantly higher than 400 mg are not recommended as they can. . . In life-threatening cases, the route of administration could also include sites other than the infected gland so as to achieve **systemic** delivery, i.e., **intravenous**, subcutaneous, or intramuscular, and rectal or oral administration of suitably encapsulated formulations in which the **lysostaphin** is protected from inactivation in the gut.

DETD It has also been found that infusion of a combination of **lysostaphin** and penicillin is surprisingly much more efficacious than **lysostaphin** alone because of an apparent synergistically enhanced bactericidal activity of this combination. In addition, it is believed that the therapeutic **lysostaphin** formulation may also include other agents which potentiate the bactericidal activity of **lysostaphin**, for example, synthetic penicillins and other antibiotics, chelating agents, mild surfactants, (e.g., deoxycholate) and other membrane active agents which may facilitate penetration of **lysostaphin** to the site of infection. In formulations that include e.g., penicillin, the dosage of **lysostaphin** can be decreased as a result of the potentiated bactericidal activity of **lysostaphin**. Since too high a dose of **lysostaphin** can induce unwanted and potentially adverse side-effects, this synergistic effect is significant not only for efficacy but also for avoidance. . .

DETD In vitro experiments were conducted to determine the bactericidal activity of **lysostaphin**, mutanolysin, and lysozyme compositions toward S. aureus and other mastitis pathogens. The protocol was as follows:

DETD . . . suspension and 1 ml of control and teat dip test formulation (i.e. milk, buffer, or buffered detergent etc., containing the **lysostaphin** composition) were combined.

DETD The results of in vitro experiments demonstrating the bactericidal efficacy of various **lysostaphin** therapeutic formulations are presented in Tables IA-IC. The results are presented as the percent survivals for S. aureus strains Newbould. . .

DETD Table IA presents results -for formulations containing 1 .mu.g/ml, 0.1 .mu.g/ml, 0.01 .mu.g/ml and 0.00 .mu.g/ml (CNTRL) **lysostaphin**. As can be seen from these results all levels of **lysostaphin** tested were effective to kill the organisms in a buffer vehicle (50 mM Tris, pH 8.0). In a milk vehicle,. . .

DETD Table IB shows the effect of adding a mild non-ionic surfactant, octylphenoxyl polyethoxy (10) ethanol, (Triton X-100), to the **lysostaphin** formulation. For example, less than 0.001% of the cells survive exposure to 0.1 .mu.g/ml **lysostaphin** and 0.1% Triton X-100, while 2.2% and 7.7%, respectively, survived exposure to each compound alone. Even more surprising, less than 0.001% survival was observed for 0.01 .mu.g/ml **lysostaphin** and 0.1% Triton X-100.

DETD Table IC demonstrates the synergistic effect of **lysostaphin** /penicillin combinations on three strains of staphylococci. Depending on the doses of each, the combinations of **lysostaphin** plus penicillin can be 100 to 1000 times more effective than either **lysostaphin** or penicillin alone with all three strains.

DETD Table ID demonstrates the effect of the combination of **lysostaphin** and penicillin compared with their sequential effect on S. aureus. S. aureus were suspended at 10.sup.7 cells/ml in milk and incubated for the times indicated in the table with either **lysostaphin** and penicillin together or sequentially. After incubation, samples were centrifuged to obtain cell pellets which were washed twice, resuspended in. . . forming units (CFU) were scored after incubation overnight at 37.degree. C. to determine percent survival relative to appropriate controls. The **lysostaphin** /penicillin combination, exhibits a synergistically enhanced bactericidal activity against S. aureus which is at least 3 orders of magnitude greater than. . .

DETD TABLE IA

The Effect of **Lysostaphin** On The Viability of S. Aureus

Strain	Vehicle	Incubation Time	% Survival 1.0L	0.1L	0.01L	CNTRL
S. aureus						
	Milk	15'	2.8	75.0	100. . .	

DETD TABLE IB

The Effect Of Non-Ionic Detergent On The Bactericidal Activity of **Lysostaphin** Toward S. aureus

% Survival

Incubation 0.1L +

0.01L +

Strain

Vehicle

Time 0.1L

0.01L

0.1%T

0.1%T
0.1%T
CNTRL

S. aureus
Buffer. . .

DETD TABLE IC

The Effect Of Penicillin On The Bactericidal
Activity of **Lysostaphin** Toward S. aureus

Strain	Vehicle	Incubation Time	% Survival 0.1L	0.01L	0.1P	0.1L + 0.1P	0.01L + 0.1P	CNTRL
S. aureus	Milk	. . .						

DETD TABLE ID

A Comparison of the Effect of the Combination of **Lysostaphin** and Penicillin Versus Their Sequential Effects on the Survival of Staphylcoccus aureus (Strain RN451) in milk at 37.degree. C.

	combo(2 h)	lspn(2 h)	pen(2 h)	Pen(2 h)/ lspn(0.5 h)	lspn(2 h)/ pen(0.5 h)
% survival	0.0005	23	25	0.3	10

lspn = **lysostaphin**; pen = penicillin

DETD In addition, assays for **lysostaphin**, mutanolysin, and lysozyme activities which measure the decrease in turbidity at 600 nm of suspensions of live S. aureus, S.. . .

DETD The data indicate that **lysostaphin** is a rapidly acting, highly effective staphylocide, the bactericidal activity of which is potentiated more than 1000 times by penicillin or the mild surfactant, Triton X-100. The inclusion of a chelating agent further potentiates the bactericidal activity of **lysostaphin**. It is also believed that synthetic penicillins and cell wall-active antibiotics will potentiate the activity of **lysostaphin**. **Lysostaphin** is an effective staphylocide in milk, but in buffer the bactericidal activity of **lysostaphin** is approximately 10 times that observed in milk.

DETD . . . . the general protocol described in Examples 1-4, further in vitro experiments were performed to evaluate the bactericidal activity of a **lysostaphin** composition comprising bacteriolytic enzymes, a non-ionic detergent and buffered chelating agent. As shown in Table II a formulation containing 1% Triton X-100, 0.1 .mu.g/ml **lysostaphin**, 10 .mu.g/ml lysozyme, and 5 mM EDTA in 20 mM Tris,

pH 8.0, (AMBI Teat Dip-0.1) was extremely effective against. . .

DETD Trials on cows were performed which demonstrated the efficacy of **lysostaphin** teat-dip compositions in vivo. The tests were performed generally according to Protocol A of the National Mastitis Council. In general,. . . and allowed to air dry for 30 minutes. Two teats (right fore and left rear) were then dipped in a **lysostaphin** test teat dip formulation (10 .mu.g/ml **lysostaphin** in 0.85% saline) to cover 2/3 of the teat., and allowed to air dry for 30 minutes; the remaining two. . .

DETD Ten .mu.g/ml solutions of **lysostaphin** in 0.85% saline completely disinfected invading S. aureus from cow teat surfaces. Moreover, **lysostaphin** applied to teat surfaces prior to exposure of teats to S. aureus suspensions had sufficient residual activity on the teat. . . of the teat. Residual activity could be enhanced by inclusion of a polymeric adsorbent and/or inert carrier protein to reduce **lysostaphin** wash-off.

DETD . . . Example 6 and the data obtained in vitro, an enhanced teat dip formulation (AMBI Teat Dip 1.0) comprising 1.0 .mu.g/ml **lysostaphin**, 10 .mu.g/ml lysozyme, 1.0 % Triton X-100, and 5 mM EDTA in 20 mM Tris buffer, pH 8.0 was evaluated. . . to air dry for 30 min. The treated teats were then dipped in AMBI test teat dip-1.0 solution (1.0 ug/ml **lysostaphin**, 10.0 .mu.g/ml lysozyme, 1.0% Triton X-100, 5 mM EDTA, 20 mM Tris buffer, pH 8.0) and allowed to air dry. . .

DETD . . . 200-300 CFU of S. aureus strain Newbould 305. Three days post-infection, the glands were infused with a single dose of **lysostaphin** dissolved in 200 .mu.l 0.85% sterile saline. Milk samples were collected from the glands 6 hours after treatment and at. . . were plated on blood agar. After 24-48 hours incubation, the plates were counted to determine CFU. The single doses of **lysostaphin** which were sufficient to eliminate the infection did not produce adverse side effects and indicated that intramammary infusions of **lysostaphin** are effective against staphylococcal mastitis. At 125 .mu.g/kg, glands were cleared of infection by the 6 hour post-treatment sample and. . .

DETD TABLE IV

Efficacy of Intramammary Infusion of **Lysostaphin** Toward Experimental STAPHYLOCOCCAL Mastitis in Guinea Pig

	Lysostaphin Dose .mu.g/kg					
	ZERO	1.0	5.0	25.0	62.5	125.0
Number of animals cleared of infection	(0/10)	(1/0)	(1/2)	(2/2)	(1/1)	(7/7)

DETD It can be seen from these examples that **lysostaphin** is effective for treatment of staphylococcal mastitis and that its effect is greatly enhanced when used in combination with penicillin. . .

DETD Production of **Lysostaphin** from Bacillus

DETD **Lysostaphin** for use according to the claimed invention can be obtained from either natural or recombinant sources. Preferably, the **lysostaphin** is obtained from cultures derived from Bacillus sphaericus strain 00 transformed by recombinant plasmids which direct **lysostaphin** synthesis as described in copending application Ser. No. 034,464 filed Apr. 10, 1987 which is a continuation-in-part of Ser. No. 852,407 filed Apr. 16, 1986. This method provides for both high levels of **lysostaphin** production substantially free from staphyloccal immunogenic contaminants. **Lysostaphin** purification is facilitated since active **lysostaphin**

accumulates directly in the growth mediums Using this method, Bacillus sphaericus 00 transformants containing plasmid PBC16-1L (B. sphaericus 00/pBC16-1L) have. . . found to be particularly suited for the purpose, although other transformed Bacillus strains are also useful as a source of **lysostaphin**.

DETD The **lysostaphin**-producing organism is grown under conditions conducive to the production of **lysostaphin**. The optimum conditions will vary from strain to strain; however, certain types of growth media and fermentation conditions are known to enhance **lysostaphin** production. In the case of the Bacillus sphaericus 00/pBC16-1L transformant, the preferred growth medium is VY broth (25 g Veal. . .

DETD TABLE V

Effect of Aeration on **Lysostaphin** Production by the Bacillus Sphaericus 00/pBC 16-1L Transformant

	Stirring Speed			
Klett	100 rpm	200 rpm	200 rpm (Fluted)	320 rpm
250	21.8	36.2. . .		

DETD Samples were removed at times throughout growth. Supernatants were assayed for **lysostaphin** activity by turbidometric clearing of dead cell suspensions of S. aureus. Results are presented as .mu.g **lysostaphin** per ml. B. sphaericus 00/pBC16-1L transformant grown on VY medium produced and secreted approximately 130 mg **lysostaphin** per liter of culture medium, which is more than four times the amount produced by S. simulans under the best fermentation conditions currently available. **Lysostaphin** accumulates in the growth medium with little or no degradation, even after prolonged incubation of cultures, and accounts for more. . .

DETD **Lysostaphin** is isolated from the growth medium in accordance with known fractional precipitation (salting out) procedures. Alternatively, a particularly effective purification is achieved by combining a precipitation and a chromatographic separation of the fermentation broth from cultures of the **lysostaphin**-producing B. sphaericus 00/pBC16-1L transformant.

DETD . . . ammonium sulfate is added to the supernatant to 40-60%, preferably 50%, of saturation. After 1 hour at 4.degree. C., the **lysostaphin**-containing precipitate is recovered by centrifugation. Recovery at this step is greater than 80%.

DETD . . . FPLC Mono S) and eluted using a buffered gradient of increasing salt concentration from 0.05 to 0.25M NaCl. Recovery of **lysostaphin** for the single chromatographic step was more than 90%. **Lysostaphin** activity is associated with two major peaks (FIG. 1). The later eluting peak of **lysostaphin** is comprised of non-covalent aggregates of the protein. These aggregates dissociate on dilution in buffer and under conditions of sodium. . .

DETD Construction of the plasmid vector pBC16-1L which contains the gene coding for **lysostaphin**

DETD **Lysostaphin**-producing strains of Bacillus sphaericus can be produced using recombinant DNA techniques and preferably those described in copending applications 852,407 and. . . gene (i.e. .beta.-galactosidase gene). The ligation mix is then transferred to E. coli (JM105) by transformation. Successful insertions of the **lysostaphin** gene into the plasmid can be found by selecting for transformants by growth on the appropriate antibiotic, and then finding those with a lac Z' negative phenotype. **Lysostaphin** production is detected by turbidometric clearing of a suspension of S. aureus either in solution format or as an overlay. . .

DETD Using various **lysostaphin**-producing E. coli JM105

transformants, restriction analysis and subcloning of the JM105 plasmid DNA showed that the DNA sequence coding for **lysostaphin** was localized to a 1.5 kbp Hpa II-Hind III DNA fragment. This fragment was visualized after electrophoresis by ethidium bromide. . . Tris, 1 mM EDTA, pH 8.0). Recombinant plasmids capable of transforming B. subtilis as well as B. sphaericus to express **lysostaphin** were constructed using a derivative of plasmid pBC16 (pBC16-1) as a cloning vector. pBC16 is a tetracycline resistant (Tet .sup.r). . .

DETD . . . The Pvu II-digested vector pBC16-1 was treated with calf intestinal alkaline phosphatase. The 1.5 Kbp DNA fragment which codes for **lysostaphin** was treated with the Klenow fragment of DNA polymerase. The 1.5 Kbp DNA fragment and plasmid DNA were then mixed. . . ligation mixture was transferred to B. subtilis by protoplast transformation. Transformants were resistant to erythromycin, sensitive to tetracycline, and produced **lysostaphin** as indicated by zones of clearing when grown on agar containing dead S. aureus cells. One such **lysostaphin** producing clone was picked and designated B. subtilis/pBC16-1L. Plasmid pBC16-1L DNA extracted from the B. subtilis/pBC16-1L transformant was isolated after. . . by protoplast transformation to various species of Bacillus, including B. sphaericus strain 00. Transformants were resistant to erythromycin and produced **lysostaphin**. The B. sphaericus 00/pBC16-1L transformant provides maximum production of **lysostaphin** and permit accumulation of intact, enzymically active product. B. sphaericus strain 00 was originally isolated from soil and is maintained. . .

CLM What is claimed is:

. . . intracellular Staphylococcus aureus comprising administering to an infected gland by intramammary infusion a therapeutic agent consisting essentially of the bacteriocin **lysostaphin** produced by recombinant means in a pharmaceutically acceptable carrier in an amount effective to eliminate the recurring staphylococcal mastitis.

2. A method according to claim 1, wherein from 2 mg to 400 mg of **lysostaphin** is administered to a bovine mammary gland.

. . . wherein the therapeutic agent further comprises a mild surfactant in an amount effective to potentiate the therapeutic effect of the **lysostaphin**.

. . . according to claim 1, wherein the therapeutic agent further comprises at least one agent which potentiates the bactericidal activity of **lysostaphin** selected from the group consisting of penicillin, synthetic penicillins, bacitracin, methicillin, cephalosporin, polymyxin and chelating agents in an amount effective to synergistically enhance the therapeutic effect of the **lysostaphin**.

. . . wherein the therapeutic agent further comprises a mild surfactant in an amount effective to potentiate the therapeutic effect of the **lysostaphin**.

L2 ANSWER 29 OF 34 USPATFULL
AN 93:65157 USPATFULL
TI Method for the prevention and treatment of bovine mastitis
IN Sordillo, Lorraine M., Saskatoon, Canada
Babiuk, Lorne A., Saskatoon, Canada
PA Ciba-Geigy Corporation, Ardsley, NY, United States (U.S. corporation)
PI US 5234684 19930810
AI US 1991-751181 19910828 (7)
DCD 20090623
RLI Continuation of Ser. No. US 1989-426287, filed on 24 Oct 1989, now patented, Pat. No. US 5124145

DT   Utility
FS   Granted
EXNAM Primary Examiner: Schain, Howard E.
LREP   Morrison & Foerster
CLMN   Number of Claims: 3
ECL   Exemplary Claim: 1
DRWN   5 Drawing Figure(s); 4 Drawing Page(s)
LN.CNT 583
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
DETD   . . . interferons of the present invention are administered by intramammary injection; however, effective dosages may be administered via intramuscular, subcutaneous, or **intravenous** injection. When prepared as injectables, the interferons are generally administered using a pharmaceutically acceptable vehicle or excipient. Suitable vehicles are,. . .
DETD   . . . (10 min, 37.degree. C.) in a shaking water bath. After initial incubation, the mixtures were washed, resuspended in HBSS containing **lysostaphin** (Sigma Chemical Co., St. Louis, Mo.), and incubated for 30 min to remove extracellular bacteria. Cells were washed and divided. . .
DETD   . . . milk but no quarter swelling; 4 is abnormal milk and swollen and/or tender quarter; and 5 is acute mastitis with **systemic** involvement.
CLM   What is claimed is:
. . . A method for treating or preventing coliform mastitis in a cow comprising administering to said cow by intramuscular, subcutaneous, or **intravenous** injection, a therapeutically effective amount of bovine interferon-.lambda., wherein said injection is given during the postpartum period.

L2   ANSWER 33 OF 34 USPATFULL
AN   88:72431 USPATFULL
TI   Particulate composition and use thereof as antimicrobial agent
IN   Violante, Michael R., Rochester, NY, United States
       Steigbigel, Roy T., Miller Pl., NY, United States
PA   University of Rochester, Rochester, NY, United States (U.S. corporation)
PI   US 4783484   19881108
AI   US 1984-658153   19841005 (6)
DT   Utility
FS   Granted
EXNAM Primary Examiner: Brown, J. R.; Assistant Examiner: Rollins, Jr., John W.
LREP   Kenyon & Kenyon
CLMN   Number of Claims: 29
ECL   Exemplary Claim: 1
DRWN   No Drawings
LN.CNT 899
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
DETD   [Methyl-.sup.3 H]thymidine with a specific activity of 5 Ci/mM was used at a concentration of 1 mCi/ml. **Lysostaphin**, with a specific activity of 240 U/ml, was diluted in PBS so that 1 ml contained 10 u of activity.. . .
DETD   . . . leukocytes were centrifuged at 200 g for 10 minutes, decanted, and the pellet resuspended in PBS containing 10 U/ml of **lysostaphin** for lysis of the remaining extracellular Staphylococci. After 10 minutes in 37.degree. C. water bath, tubes were centrifuged 10 minutes. . . without IEE particles, was centrifuged at 1100 g for 20 mins, decanted, and the pellet resuspended in 1 ml of **lysostaphin** as a control of **lysostaphin** activity, or 1 ml of PBS as a growth control.
DETD   . . . demonstrated a 20 percent survival rate (LD.sub.80) after 10

days. A second group of ten mice were given a single **intravenous** injection of one micron iodipamide ethyl ester particles 90 minutes after the S. aureus injection. The IEE particle dose was. . .